UNIVERSITY OF ALBERTA

POSSIBLE ROLES FOR THE VACUOLAR H^+-ATPASE AND F_1F_0-ATPASE IN ALUMINUM RESISTANCE

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled POSSIBLE ROLES FOR THE VACUOLAR H^+-ATPASE AND F_1F_0-ATPASE IN ALUMINUM RESISTANCE submitted by CHRISTIE ANNE HAMILTON in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Molecular Biology and Genetics.

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ABSTRACT

Crop yields are significantly reduced under acidic soil conditions, where the speciation of aluminum (Al) compounds changes so that Al becomes soluble and/or toxic. Recent research has focused on developing crop cultivars resistant to soluble Al in the soil. Previous research in our laboratory identified two 51 kD, Al-induced proteins from an Al-resistant cultivar of wheat (*Triticum aestivum* L.).

This thesis describes the purification and identification of the 51 kD, aluminum-induced proteins from wheat. Peptide microsequence analysis revealed that the purified peptides were homologous to subunits of the vacuolar H^+-ATPase (V-ATPase) and the mitochondrial ATP synthase (F_1F_0-ATPase). Both V-ATPase and F_1F_0-ATPase activities were induced by Al in the Al-resistant cultivar, PT741, and not in other cultivars tested. Based on these results, I hypothesized that increased V-ATPase activity could contribute to Al resistance by counteracting cytoplasmic acidosis caused by Al-inhibition of the plasma membrane ATPase. Vacuolar ATPase activity could maintain cytoplasmic pH homeostasis by transporting protons into the vacuole. Alternatively, it may be required to allow tonoplast transmembrane transport of other molecules, such as organic acids. The ATP required to fuel this process could be provided by the mitochondrial ATP synthase.

This hypothesis was tested in the yeast and *Arabidopsis* model systems. Yeast V-ATPase mutant strains were hypersensitive to Al, while F_1F_0-ATPase mutant strains exhibited wild-type growth. Since the tonoplast membrane potential generated by V-ATPase activity is also thought to be required for NaCl tolerance in yeast, the scope of my research was expanded to include analysis of the yeast response to NaCl.

As was observed with Al, V-ATPase mutant strains were hypersensitive to NaCl while F_1F_0-ATPase mutant strains exhibited wild-type growth. Transgenic *Arabidopsis* expressing a cDNA encoding the B subunit of the V-ATPase in the antisense orientation were also hypersensitive to Al and, to a lesser extent, NaCl.

In conclusion, this research describes the development and testing of a novel hypothesis that describes potential roles for the vacuolar H^+-ATPase and the F_1F_0-ATPase in Al and NaCl tolerance. Determination of the precise mechanism by which increased V-ATPase activity mediates Al and NaCl tolerance awaits future investigation.

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1. Introduction

1.1 Agricultural Significance of Aluminum

Crop yields are significantly reduced under acidic soil conditions, where the speciation of aluminum (Al) naturally present in soil changes such that Al becomes soluble and toxic. Acidic soils comprise approximately 40% of the world's arable land, and this area (Fig 1-1) is likely expanding due to acidic precipitation, use of nitrogenous fertilizers and poor land management practices in newly cleared tropical forests (von Euxkull and Mutert, 1995). The poor fertility of acidic soils is aggravated by physical factors such as low water-holding capacity, susceptibility to crusting, erosion and compaction (von Euxkull and Mutert, 1995). Historically, Al toxicity has been ameliorated by liming soil. This increases soil pH so that Al becomes insoluble and/or nontoxic. Since this method is expensive and fails to correct subsoil acidity, recent research has focused on developing crop cultivars resistant to soluble Al in soil.

1.2 Effects of Aluminum on Plant Cellular Metabolism

Aluminum disturbs cellular metabolism and inhibits growth of Al-sensitive plants. Decreased growth correlates with inhibition of cell division (Morimura *et al.*, 1978) and elongation (Wallace and Anderson, 1984; Taylor and Foy, 1985), reduced mineral uptake by roots (Baligar *et al.*, 1987), decreased activity of calmodulin-

Figure 1-1. Global distribution of acidic soils (indicated in red). For the purpose of this figure, topsoils of pH 5.5 or less are considered significantly acidic. (Adapted from van Wambeke, 1976; Wright *et al.* (eds), 1990)

regulated enzymes (Putterill and Gardner, 1988; Siegel and Haug, 1983, Suhayda and Haug, 1985; Slaski, 1989), reduced DNA synthesis (Matsumoto and Morimura, 1980; Wallace and Anderson, 1984) and disruption of the Golgi apparatus in root cap cells (Bennet *et al.*, 1987).

1.3 The Search for a Primary Toxic Lesion

Recent research has attempted to identify the primary toxic lesion caused by Al. However, the multitude of symptoms exhibited by plants grown in the presence of Al has made it difficult to determine a single, primary cause for growth inhibition. One mechanism that might account for the range of symptoms observed is interaction of Al with calcium (Taylor, 1988; Rengel, 1992; Kochian, 1995). Aluminum interferes with transport of calcium (Ca^{2+}) across the plasma membrane by blocking Ca^{2+} channels (Ding *et al.*, 1993; Pineros and Tester, 1995). However, this is unlikely to be directly responsible for Al toxicity, since root growth can be inhibited at low Al activities without affecting Ca^{2+} uptake (Ryan *et al.*, 1997). Aluminum also interacts with calcium by disrupting Ca^{2+} homeostasis in the cytoplasm, either by affecting its activity (Nichol *et al.*, 1993) or by interfering with its role as a secondary messenger, perhaps through the inositol 1,4,5-triphosphate pathway (Jones and Kochian, 1995). In *Arabidopsis*, Al exposure results in prolonged elevation in cytoplasmic Ca^{2+}, which occurs after growth inhibition (Jones *et al.*, 1998a). This suggests that alteration of Ca^{2+} homeostasis is not a required event for Al toxicity. In contrast, exposure to toxic concentrations of Al causes a rapid reduction in cytoplasmic Ca^{2+} in cultured tobacco

cells, which correlates with growth inhibition (Jones *et al.*, 1998b). To address this contradictory data, Plieth *et al.* (1999) constructed transgenic *Arabidopsis* expressing the calcium-activated photoprotein, aequorin, to allow measurement of Ca^{2+} in intact whole roots exposed to Al. Their results suggest that by blocking Ca^{2+} channels, Al prevents Ca^{2+} influx and makes plants vulnerable to low pH (Plieth *et al.*, 1999). However, their model cannot account for the ability of other cations to ameliorate Al toxicity (Ryan *et al.*, 1997). Aluminum could also exert its toxic effect by displacing Ca^{2+} from critical sites in the apoplasm, where Ca^{2+} normally binds to negatively charged proteins and pectins in the cell wall (Hanson, 1984; Kinraide and Parker, 1987; Kinraide *et al.*, 1994). However, this hypothesis is not supported by the observation that other cations can alleviate Al stress, some even more effectively than added Ca^{2+} (Ryan *et al.*, 1997). A number of other lesions, such as peroxidation of membrane lipids, have also been proposed as possible primary toxic lesions (Yoshino *et al.*,1999). However, it is perhaps unlikely that a single primary toxic lesion exists due to the extreme reactivity of Al with cellular components.

1.4 Genetic Variation in Aluminum Tolerance

Many species exhibit genetic variability in their responses to Al stress. Although Al-resistant cultivars of wheat and other crop plants have been developed and studied (reviewed by Carver and Ownby, 1995), it is difficult to separate the effects of different resistance mechanisms due to their possible interactions and compensatory effects. In barley (*Hordeum vulgare*), evaluation of 37 genotypes of

diverse origin suggested that Al tolerance is controlled by a single locus with multiple alleles (Minella and Sorrels, 1992). Studies using other genotypes suggested that at least two dominant, independent loci are involved in the Al tolerance phenotype (Gallego and Benito, 1997). Although originally thought to be controlled by a single gene (*Alm1*, Moon *et al.*, 1997), Al tolerance in maize (*Zea mays*), was later shown to be controlled by two loci, one located on chromosome 6 and the other on chromosome 10 (Sibov *et al.*, 1999). In rye (*Secale cereale* L.), Al tolerance is controlled by at least two independent and dominant loci (*Alt1* and *Alt3*) located on chromosomes 6RS and 4R (Gallego *et al.*, 1998). In some wheat genotypes (for example Atlikat) aluminum tolerance behaves as a single gene trait in crosses (Basu *et al.*, 1997). However, many different genes have been implicated in other populations, suggesting that Al tolerance in wheat is a dominant trait controlled by several genes (Aniol, 1991; Berzonsky, 1992). The genetic basis of Al resistance in dicots has not been extensively studied, but in soybean (*Glycine max*), it is associated with five quantitative trait loci (Bianchi-Hall *et al.*, 2000). The genetic complexity associated with Al tolerance makes it difficult to understand the contributions of different components. However, this very complexity is a potentially valuable resource for breeding programs to increase the Al tolerance of locally adapted commercial cultivars. Furthermore, molecular techniques have provided new tools to separate different components of Al resistance so that they can be studied in isolation.

1.5 Mechanisms of Aluminum Resistance

Aluminum resistance may be mediated by both internal and external mechanisms. External mechanisms detoxify Al in the apoplasm or prevent it from crossing the plasma membrane to reach sensitive cytoplasmic sites, while internal mechanisms detoxify Al once it has crossed the plasma membrane, either by immobilization or sequestration (Taylor, 1991).

1.5.1. Al-induced Release of Organic Anions

One example of an external Al resistance mechanism is Al-induced release of organic anions, which chelate Al in the apoplasm and limit its entry into the symplasm. Increased exudation of organic anions such as citrate and malate correlates with increased Al resistance in snapbean (Miyasaka *et al.*, 1991), wheat (Delhaize *et al.*, 1993; Ryan *et al.*, 1994; Basu *et al.*, 1994c) and maize (Pellet *et al.*, 1994). Addition of malate to the growth medium restores root elongation to control levels in an Al-sensitive cultivar of wheat (Basu *et al.*, 1994c), suggesting that malate plays a role in Al resistance. The role of organic anions in Al tolerance has been investigated directly using transgenic plants. Overexpression of citrate synthase in transgenic tobacco (*Nicotiana tabacum*) and papaya (*Carica papaya*) caused increased citrate efflux, which correlated with Al tolerance (de la Fuente *et al.*, 1997). This interesting result was not reproducible, however, since subsequent studies using independent lines of transgenic tobacco found that overexpression of citrate synthase had no effect on citrate efflux levels or Al tolerance (Delhaize *et al.*, 2001). Since

organic acids exist as organic acid anions in the cytoplasm, involvement of Al-activated anion channels in Al resistance has been hypothesized (Delhaize and Ryan, 1994). This is supported by evidence showing that anion channel antagonists block Al-induced efflux of malate in an Al-tolerant cultivar of wheat (Ryan *et al.*, 1994). Protein kinase inhibitors can also inhibit malate efflux, suggesting that activation of an Al-activated plasma membrane anion channel may be controlled by protein phosphorylation (Osawa and Matsumoto, 2001). Aluminum-activated, malate-permeable channels have been characterized using the patch-clamp technique in maize and wheat (Pineros and Kochian, 2001; Zhang *et al.*, 2001). Continuing research in this area has focused on identification of these channels.

1.5.2 Plant-Induced Elevation of Rhizosphere pH

A second mechanism of external Al resistance is plant-induced elevation of rhizosphere pH. This is an attractive hypothesis because the chemistry of Al favours formation of insoluble and/or nontoxic Al species at near neutral pH. Early studies using 20 winter and 20 spring wheat cultivars observed a negative correlation between cultivar tolerance to Al and pH changes of nutrient solutions (Taylor and Foy, 1985 a,b). These pH differences were caused by differences in ammonium and nitrate nutrition (Taylor and Foy, 1985c), but subsequent studies found that the relative tolerance of Atlas-66 (Al-resistant) and Scout-66 (Al-sensitive) was unaffected by the ratio of ammonium:nitrate and by solution pH (Taylor, 1987). There were also no discernable differences in the rhizospere pH of Atlas-66 and

Scout-66, as measured with pH microelectrodes (Miyasaka *et al.*, 1989). However, recent experiments suggest that plant-induced elevation of rhizosphere pH may occur in *Arabidopsis*. An Al-resistant mutant exhibits an Al-induced increase in rhizosphere pH mediated by increased net proton influx into the cytoplasm (Degenhardt *et al.*, 1998).

1.5.3 Exudation of Al-binding Polypeptides

A third mechanism of external Al resistance is the exudation of polypeptides which may bind Al or counteract its growth-inhibitory effects in the apoplasm. Aluminum-resistant and Al-sensitive cultivars of wheat show differences in polypeptide exudation by roots under conditions of Al stress (Delhaize *et al.*, 1990). In Al-resistant cultivars, specific polypeptides accumulate in exudates and have greater association with Al than those exuded by sensitive cultivars. This implies that polypeptides specifically produced by Al-resistant cultivars may facilitate external Al resistance (Basu *et al.*, 1994b, 1997), although the physiological significance of most of these proteins has not yet been ascertained. A 23 kD root exudate protein from an Al-resistant isoline of wheat (Alikat = Katepwa*3/Maringa) is induced by Al treatment, binds Al and segregates with Al resistance in the progeny of a cross between doubled-haploid lines of Alikat (Al-resistant) and Katepwa (Al-sensitive). This suggests that this 23 kD protein is involved in mediating Al resistance (Basu *et al.*, 1997). The purified protein has been identified as an apoplastic form of

superoxide dismutase, and may be involved in preventing oxidative damage caused by Al (Basu *et al.*, 2001).

1.5.4 Support for External Resistance from Inhibitor Studies

The involvement of external resistance mechanisms, such as organic anion and polypeptide export, is supported by the effects of biological inhibitors on Al uptake. Excised roots from Al-resistant wheat cultivars show increased net Al uptake when exposed to dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. A similar effect has been observed in intact single cells of the giant algae, *Chara corallina*, where treatment with DNP causes increased net Al uptake into the protoplasm (Taylor *et al.*, 2000). Based on these observations, it appears that an energy-dependent exclusion mechanism is involved in Al-resistance, perhaps mediated by exudation of chelator ligands such as malate and/or polypeptides to the rhizosphere (Zhang and Taylor, 1991). Cycloheximide, an inhibitor of cytplasmic protein synthesis, decreases Al uptake by Al-sensitive cultivars, but not by resistant cultivars, suggesting that proteins encoded by nuclear genes might be involved in regulating uptake of Al across the plasma membrane and may play a role in Al susceptibility (Zhang *et al.*, 1995).

1.5.5 Modulation of Intracellular Enzyme Activity

Observations of changing intracellular enzyme activity support the involvement of internal mechanisms in Al resistance. Activities of intracellular

enzymes such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Sasaki *et al.*, 1996) and vacuolar H^+-ATPase (Kasai *et al.*, 1992, 1993; Hamilton *et al.*, 2001) are stimulated by Al. Transcripts encoding other intracellular proteins such as glutathione S-transferase (Richards *et al.*, 1998), and a fimbrin-like protein (Cruz-Ortega *et al.*, 1997) from wheat, and transcripts encoding aldolase, peroxidase, glutathione S-transferase, blue-copper-binding protein, superoxide dismutase and a reticuline:oxygen oxidoreductase homolog from *Arabidopsis* are also induced by Al (Richards *et al.*, 1998). Most of these Al-induced gene products (Table 1-1) appear to be involved in general stress response, since they are also induced by oxidative stress (Richards *et al.*, 1998), other toxic metals, low calcium levels and wounding (Snowden *et al.*, 1995; Hamel *et al.*, 1998). The involvement of blue-copper-binding protein, glutathione S-transferase, peroxidase and GDP-dissociation inhibitor in Al resistance has been confirmed in transgenic *Arabidopsis*, where their overexpression confers increased Al resistance (Ezaki *et al.*, 2000).

1.5.6 Sequestration of Al in the Vacuole

Perhaps the most compelling example of a possible internal Al resistance mechanism is sequestration of Al in the vacuole, where it would likely be chelated by organic acids or phosphates. Although measurement of intracellular Al is technically difficult, Al uptake across the plasma membrane has been arguably demonstrated in

Table 1-1. Classification of Al-induced genes.

Gene	Identity	Species	Reference
Pathogenesis-Related			
Wali5	Bowman-Birk protease inhibitor	*T. aestivum*	Snowden and Gardner, 1993
AtBP1	Bowman-Birk protease inhibitor	*A. thaliana*	Richards *et al.*, 1997
Oxidative Stress-Related			
AtBCB	Blue copper binding protein	*A. thaliana*	Richards *et al.*, 1997
AtPOX	Peroxidase	*A. thaliana*	Richards *et al.*, 1997
AtSOD	Superoxide dismutase	*A. thaliana*	Richards *et al.*, 1997
TaSOD	Superoxide dismutase	*T. aestivum*	Basu *et al.*, 2001
	Reticuline:oxygen oxidoreductase	*A. thaliana*	Richards *et al.*, 1997
NtPOX	Peroxidase	*N. tabacum*	Ezaki *et al.*, 1995
ParB	Glutathione S-transferase	*N. tabacum*	Ezaki *et al.*, 1995
General Stress-Related			
HSP150	Heat shock protein	*S. cerevisiae*	Ezaki *et al.*, 1998
Unknown Function			
SED1	Putative membrane protein	*S. cerevisiae*	Ezaki *et al.*, 1998
ParA	No homologs	*N. tabacum*	Ezaki *et al.*, 1995
Wali1	Homology to metallothioneins	*T. aestivum*	Snowden and Gardner, 1993
Wali2	No Homologs	*T. aestivum*	Snowden and Gardner, 1993
Wali3	No Homologs	*T. aestivum*	Snowden and Gardner, 1993
Wali4	Phenylalanine ammonia lyase	*T. aestivum*	Snowden and Gardner, 1993
Wali6		*T. aestivum*	Snowden and Gardner, 1994
Wali7		*T. aestivum*	Snowden and Gardner, 1994
	Fimbrin-like protein	*T. aestivum*	Cruz-Ortega *et al.*, 1997
	Aldolase	*A. thaliana*	Richards *et al.*, 1998

Saccharomyces cerevisiae (Ezaki *et al.*, 1999; Jagannatha Rao *et al.*, 1997), *Triticum aestivum* (Zhang and Taylor, 1989), *Phaseolus vulgaris* (McDonald-Stephens and Taylor, 1995), *Glycine max* (Lazof *et al.*, 1994) and the algae *Chara corallina* (Taylor *et al.*, 2000). It is generally agreed that the majority of Al is associated with the cell wall, but it is also taken up into the vacuole (Taylor *et al.*, 2000). Based on observations using the Al-binding fluorescent dye, morin, Ezaki *et al.* (1999) suggested that intracellular Al does not associate with the yeast vacuole. However, low levels such as those measured in *Chara* (Taylor *et al.*, 2000) would not be detected using this method. The mechanism by which sequestration in the vacuole may occur is unknown, but Al may enter the vacuole nonspecifically via transporters of essential metals or as an unidentified conjugate. In either case, vacuolar ion uptake utilizes proton motive force generated primarily by the vacuolar H^+-ATPase (V-ATPase).

1.6 Structure of the Vacuolar H^+-ATPase

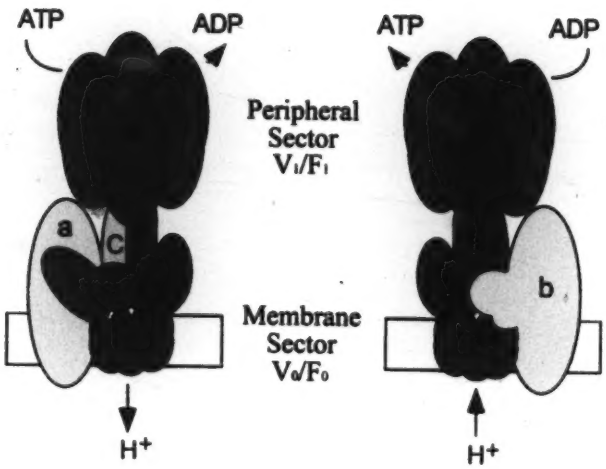
The vacuolar H^+-ATPase (V-ATPase) and the closely related mitochondrial F_1F_0-ATPase are complex, multisubunit enzymes (Fig 1-2, Table 1-2). They share both a common structure and a common mechanism of action. Their structures consist of catalytic (V_1 and F_1, respectively) and membrane sectors (V_0 and F_0,

Table 1-2. Subunit composition of the vacuolar ATPase (V-ATPase) and functional homologies to subunits of the mitochondrial ATP synthase (F_1F_0-ATPase).

V-ATPase Subunit	Functional Homolog in F_1F_0-ATPase	Function	Molecular Mass (kD)
V_1 Constituents		ATP Binding and Catalysis	500-600
A	β	ATP hydrolysis (synthesis in F_1)	67-73
B	α	ATP binding, activity regulation*	55-60
C	unknown	Assembly*	44
D	γ	Spindle*	32
E	γ	Spindle*	31
F	ε	V_1-V_0 contact, regulation*	14
G	F_0-b	Stabilization	12-16
H	unknown	V_1 stabilization	50-54
V_0 Constituents		Proton Translocation	250-300
a	b	Activity, assembly, targeting	97-115
d	unknown	Assembly, stabilization	32-38
c	c	Proton translocation	16
c′	c	Proton translocation	17
c″	c	Proton translocation	17

*These functions have been tentatively assigned based on similarity to F_1F_0-ATPases

Figure 1-2. Structural comparison of the vacuolar ATPase (V-ATPase) and mitochondrial ATP synthase (F_1F_0-ATPase). A generalized V-ATPase is shown on the left and a generalized F_1F_0-ATPase is shown on the right. The functions (if known) of each subunit are described in Table 1-2 and in the text. Adapted in part from Sze *et al.*, 1999.



V-ATPase F_1F_o-ATPase

respectively). The catalytic sectors are responsible for energy conversion, while the membrane sectors are responsible for proton transport through the membrane. The V-ATPase is localized to most membranes of the secretory pathway and is particularly abundant in the tonoplast of the plant vacuole. It functions in energizing these membranes to allow secondary active transport, and in acidifying internal compartments. The F_1F_0-ATPase is localized to the mitochondria and chloroplasts, where it functions predominantly in ATP synthesis. Interestingly, the V-ATPase is essential in all organisms tested, with the exception of yeast, while many types of cells can survive without a functional F_1F_0-ATPase (King and Attardi, 1989; Dow *et al.*, 1997; Bowman *et al.*, 2000). The characteristic phenotype of yeast mutants lacking V-ATPase activity is sensitivity to calcium in the growth medium, inability to grow in medium buffered to neutral pH and inability to utilize nonfermentable carbon sources (Graham *et al.*, 2000).

The catalytic sector of the V-ATPase consists of three copies each of the A and B subunits, which are approximately 25% identical at the amino acid level to the β and α subunits of the F_1F_0-ATPase, respectively. This implies evolution from common ancestral genes (Holzenburg *et al.*, 1993; Davies *et al.*, 1996; Bowman *et al.*, 1988; Manolson *et al.*, 1988; Nelson *et al.*, 1989). Subunit A (67-73 kD) contains the ATP hydrolytic domain (Arai *et al.*, 1987), while subunit B (55-60 kD) is responsible for ATP binding, and may be involved in regulation of V-ATPase activity (Manolson *et al.*, 1985; Zhang *et al.*, 1995; Peng, 1995). The function of Subunit C (44 kD) is not known, but it may be involved in assembly of V_1 (Ho *et al.*, 1993).

Subunits D (32 kD) and E (31 kD) show regions of similarity to the γ subunit of F_1, and may functionally associate to form a homolog of the F_1 spindle (Tomashek *et al.*, 1996). Subunit F (14 kD), analogous to the ε subunit of F_1, may have a role in stabilizing contacts between V_1 and V_0 (Graham *et al.*, 1994) or it may have a regulatory role (Nelson *et al.*, 1994). Subunit G (16 kD) shows regions of similarity to the stabilizing b subunit of F_0, although in the *Manduca sexta* midgut, it is present in three copies rather than the two copies of subunit b found in the F_1F_0-ATPase (Supekova *et al.*, 1995).

The membrane sectors of the V-ATPase and F_1F_0-ATPase (V_0 and F_0, respectively) have similar functions in proton translocation across the membrane, but V_0 has some unique properties. The proteolipid subunits (subunit c in both enzymes) are homologous, but the V_0 proteolipid is 16 kD while the F_0 proteolipid is only 8 kD (Mandel *et al.*, 1988). This difference may have arisen by gene duplication and fusion of an ancestral gene homologous to that encoding the F_0 proteolipid (Nelson, 1992). In yeast, the V_0 sector contains several copies of subunit c and one copy each of homologous proteolipids, c' and c'', all of which are required for activity (Powell *et al.*, 2000). In contrast, the F_0 sector consists of variable numbers of subunit c (Hirata *et al.*, 1997, Powell *et al.*, 2000). These differences are thought to affect protein packing within the proton channel. The channel in V_0 is wider than that in F_0, allowing for proton slip when the proton motive force (pmf, $\Delta\mu_{H+}$) becomes too great to allow further proton pumping (Perzov *et al.*, 2001).

1.7 Function of the Vacuolar H^+-ATPase

In a typical plant cell, activity of the V-ATPase, using ATP produced by the F_1F_0-ATPase and in conjunction with the vacuolar H^+-pyrophosphatase, is responsible for maintaining a proton electrochemical gradient across the membranes of endomembrane compartments. These include the endoplasmic reticulum, golgi, coated vesicles, provacuoles and vacuoles (Herman *et al.*, 1994; Robinson *et al.*, 1996). The electrochemical gradient allows secondary active transport of cationic solutes into and anionic solutes out of the vacuolar lumen. Energetically favourable transport of cations out of and anions into the lumen is carried out by electrogenic channels. Due to its wide distribution within the cell, the V-ATPase is thought to be involved in a number of cellular activities in addition to ion transport. For example, vesicle acidification is required for ligand-receptor dissociation, protein-protein interaction (Stevens and Forgac, 1997), protein trafficking (Morano and Klionsky, 1994) and membrane fusion (Ungermann *et al.*, 1998). These processes are required for processes such as vacuole biogenesis, cell division and secretion.

1.8 Control of ATPase Expression

Activities of cellular proton pumps, which use ATP generated by the F_1F_0-ATPase, are coordinated in response to growth, development and changes in the environment. For example, transport activities of the V-ATPase and plasma membrane H^+-ATPase (P-ATPase) regulate turgor pressure during growth.

Coordinated activity of these enzymes is also required to maintain pH homeostasis during pH changes in the environment (Young *et al.*, 1998). This is achieved by sequestration of protons in the vacuole by increased V-ATPase activity combined with extrusion of protons from the cytoplasm by increased P-ATPase activity. A similar mechanism is used by NaCl tolerant plants, which sequester Na^+ in the vacuole and also actively transport Na^+ out of the cell across the plasma membrane. This phenomenon has been extensively studied in the facultative halophyte, *Mesembryanthemum crystallinum* (iceplant), and in the halotolerant sugar beet, *Beta vulgaris*. In both species, V-ATPase expression is stimulated by NaCl treatment (Ratajczak *et al.*, 1994; Kirsch *et al.*, 1996). Increased activity of V-ATPase allows accumulation of Na^+ in the vacuole via the Na^+/H^+ antiport protein, NHX1. The importance of vacuolar H^+/Na^+ antiport has been confirmed by overexpressing *NHX1* in transgenic *Arabidopsis*, where it causes enhanced NaCl tolerance (Apse *et al.*, 1999). Toxic Na^+ ions are also removed from the cytoplasm by active transport through the plasma membrane Na^+ transporter, ENA1, which is energized by the plasma membrane H^+-ATPase (Garciadeblas *et al.*, 1993).

The mechanisms by which vacuolar ATPase activity is modulated by developmental stage and the environment are not clear. Short-term adjustments in activity may occur via reversible dissociation of the peripheral V_1 subunits from the V_0 sector, as has been observed during cold stress in *Vigna radiata* (Matsuura-Endo *et al.*, 1992) and during glucose deprivation in *Saccharomyces cerevisiae* (Kane, 1995). Vacuolar ATPase activity is also affected by Cl^- levels, pH and the ratio of ATP to

ADP (Dietz *et al.*, 2001), although there is no evidence for a direct regulatory role of these factors. The lipid environment also has an effect on V-ATPase activity. V-ATPase activity is stimulated by phospholipids (Yamanishi and Kasamo, 1993), while it is inhibited by glycolipids (Kasamo *et al.*, 2000). This could be caused by changes in membrane fluidity or by conformational changes in the V-ATPase (Kasamo *et al.*, 2000). The presence of additional subunits can also affect V-ATPase activity. This has been best characterized in the salt response of *M. crystallinum*, where a change in V-ATPase subunit structure (induced subunits E_i and D_i) during salt exposure correlates with increased enzyme activity (Ratajcak *et al.*, 2000). Several small proteins have also been identified as inhibitors (Zhang K *et al.*, 1992) and activators (Xie *et al.*, 1993; Zhang *et al.*, 1992) of V-ATPase activity. Interestingly, the V-ATPase also responds to the redox status of the cell. The V-ATPase A and E subunits contain cysteine residues which form intramolecular disulphide bonds under oxidizing conditions (Feng and Forgac, 1994; Dietz *et al.*, 2001) when the cytoplasm is not acidified. In the event of cytoplasmic acidosis, this inhibition cannot occur, further illustrating the importance of the V-ATPase in stress responses (Dietz *et al.*, 2001).

Long-term adjustments in V-ATPase activity are thought to be controlled at the level of gene expression, although this phenomenon has not been studied in detail. Transcript levels of the V-ATPase A subunit increase in response to salt stress in *Nicotiana tabacum* (Narasimhan *et al.*, 1991) and *Lycopersicon esculentum* (Binzel and Dunlap, 1994). In *M. crystallinum*, levels of transcripts encoding the V-ATPase

A, B, E and c subunits increase in response to salt stress (Low *et al.*, 1996; Tsiantis *et al.*, 1996; Golldack and Dietz, 2001). The effect of salt or other stresses on levels of transcripts encoding the other V-ATPase subunits has not been examined. Interestingly, levels of the transcript encoding the V-ATPase c subunit also show diurnal regulation in leaves (Low *et al.*, 1996), although under normal growth conditions levels of transcripts encoding the V-ATPase A and B subunits remain constant. It is unknown whether the above effects on V-ATPase gene expression in response to developmental and environmental changes are due to transcriptional regulation or posttranscriptional events.

1.9 Objectives

Two 51 kD microsomal proteins are induced in roots of the Al-resistant cultivar of wheat, PT741, under conditions of Al stress (Basu *et al.*, 1994a). These proteins show dose-dependent accumulation in the presence of Al and disappear within 24 h upon removal of Al stress (Basu *et al.*, 1994a). The proteins are localized at the tonoplast of root tip cells, and segregate with the resistance phenotype (Taylor *et al.*, 1997). This is an exciting result because it suggests that at least one of these proteins may be involved in Al resistance. Localization to the tonoplast also suggests that they may be involved in a sequestration mechanism of Al resistance (Taylor *et al.*, 1997).

Differential expression of these 51 kD proteins in an Al-resistant cultivar of wheat raises several questions. Does expression of the proteins contribute to the

resistance phenotype? How is expression of the proteins regulated? What would be the effect of expressing the proteins in a heterologous, Al-sensitive genotype? To address these questions, my objectives were to purify and identify the 51 kD, Al-induced proteins from a tonoplast-enriched membrane fraction of Al-resistant wheat, to evaluate expression of the proteins, and to determine the effect of altering expression of the genes encoding these proteins in a heterologous system. My hypothesis is that eliminating expression of one or both 51 kD proteins will confer Al hypersensitivity to a heterologous genotype. Understanding the function of these Al-induced proteins will add to our knowledge of Al stress physiology, with potentially significant agricultural and economic impact. If we can alter expression of the 51 kD proteins, it may be possible to produce transgenic lines of common crop plants such as canola (*Brassica napus)* and wheat showing enhanced resistance to Al. This could increase crop yield in the presence of soluble Al, and contribute to improving agriculture in areas of soil acidity.

In Chapter Two, I describe purification of the 51 kD, Al-induced proteins from an endomembrane-enriched membrane fraction isolated from an Al-resistant cultivar of *Triticum aestivum* L. (cv PT741) grown in the presence of Al. The identity of these proteins has been determined using peptide microsequence data. Purified peptides were homologous to the B subunit of the vacuolar H^+-ATPase (V-ATPase) and to the α and β subunits of F_1F_0-ATPase (mitochondrial ATP synthase). Both V-ATPase and F_1F_0-ATPase activities were induced by Al treatment in PT741 and not

in other cultivars tested. These results suggest that upregulation of ATPase activity in PT741 may be an adaptive response involved in Al resistance.

Yeast has previously been used as a model system to examine Al stress (MacDiarmid and Gardner 1996, 1998; Ezaki *et al.*, 1998, 1999). Use of yeast as a model is uniquely appropriate here, because yeast is the only eukaryote tested thus far that does not require a functional V-ATPase for growth (Dow *et al.*, 1997; Bowman *et al.*, 2000). In Chapter Three, I describe the use of the yeast model system to simply and directly test my hypothesis that upregulation of V-ATPase and F_1F_0-ATPase activity in PT741 may be an adaptive response involved in Al resistance. Increased activity of the V-ATPase, using ATP provided by the F_1F_0-ATPase, could be required to energize the tonoplast membrane, allowing transport of Al-complexing compounds such as organic acids out of the vacuole. It is also possible that increased V-ATPase activity is required to allow sequestration of Al within the vacuole. Although V-ATPase transcript and protein levels, as well as enzyme activity, are not affected by increasing Al concentrations in yeast as they are in plants, the observation that V-ATPase mutants are hypersensitive to Al supports my hypothesis. In wild-type yeast, F_1F_0-ATPase transcript, protein and activity levels all increase in response to Al, while F_1F_0-ATPase mutants do not show an Al hypersensitive phenotype.

To further examine the importance of the V-ATPase and the vacuole in metal tolerance, I investigated the effect of vacuolar ATPase mutations on salt (NaCl) tolerance in Chapter Four. Salt tolerance in yeast is a complex trait, involving regulation of membrane polarization, Na^+ efflux and sequestration of Na^+ in the

vacuole. Since transmembrane transport energized by H^+-ATPases is common to all of these tolerance mechanisms, the responses of the plasma membrane H^+-ATPase, vacuolar H^+-ATPase and mitochondrial F_1F_0-ATPase to NaCl stress were characterized. Since the vacuolar ATPase is responsible for generating the proton motive force required for import of cations (such as Na^+) into the vacuole, strains lacking this activity should be hypersensitive to NaCl. We found that strains lacking vacuolar ATPase activity were in fact hypersensitive to NaCl, while strains lacking ATP synthase were not.

My observations in wheat and yeast suggest that V-ATPase activity is required for Al and NaCl tolerance. Examining the effect of V-ATPase mutations in plants, however, would provide the most convincing test possible of our hypothesis. Therefore, in Chapter Five, I describe the construction and phenotypic analysis of transgenic *Arabidopsis thaliana* expressing a cDNA encoding the V-ATPase B subunit in the antisense orientation. Decreased V-ATPase activity caused transgenic plants to be hypersensitive to both Al and NaCl, as measured by differences in root elongation. This approach allowed me to directly test my hypothesis and further characterize the involvement of the V-ATPase in mediating Al and NaCl tolerance.

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2. Induction of Vacuolar ATPase and Mitochondrial ATP Synthase by Aluminum in an Aluminum-Resistant Cultivar of *Triticum aestivum* L.[1]

2.1 Introduction

A wide range of proteins are induced by aluminum stress in wheat and other plant species. Aluminum-induced proteins include membrane-bound (Basu *et al.*, 1994a; Taylor *et al.*, 1997; Cruz-Ortega *et al.*, 1997), cytosolic (Richards *et al.*, 1998), cytoskeletal (Cruz-Ortega *et al.*, 1997) and exudate (Basu *et al.*, 1994b, 1997) proteins. Many of these have been implicated as general stress response proteins (Snowden *et al.*, 1995; Hamel *et al.*, 1998). Others have been associated with oxidative and other stresses (Richards *et al.*, 1998; Hamel *et al.*, 1998). The potential roles of others are still unclear. However, few have been functionally characterized as to their possible roles in Al tolerance.

The majority of Al-induced gene products have been identified at the RNA level. These include the *wali* (*w*heat *al*uminum-*i*nduced) and *war* (*w*heat *a*luminum *r*egulated) gene products (Snowden and Gardner, 1993; Richards *et al.*, 1994; Hamel

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et al., 1998), β-1,3-glucanase (Cruz-Ortega *et al.*, 1997), glutathione S-transferase (Richards *et al.*, 1998), a fimbrin-like protein (Cruz-Ortega *et al.*, 1997) and a mitochondrial Mn-superoxide dismutase (Basu *et al.*, 2001) from wheat. In *Arabidopsis*, transcripts encoding aldolase, peroxidase, glutathione S-transferase, blue-copper-binding protein, superoxide dismutase and a reticuline:oxygen oxidoreductase homolog are all induced by Al (Richards *et al.*, 1998). These gene products appear to be involved in general stress response, since they are also induced by oxidative stress (Richards *et al.*, 1998), other toxic metals, low calcium levels and wounding (Snowden *et al.*, 1995, Hamel *et al.*, 1998). Recently, expression of genes encoding the *Arabidopsis* blue-copper-binding protein, and tobacco glutathione S-transferase, peroxidase and GDP-dissociation inhibitor have been shown to confer resistance to Al in transgenic *Arabidopsis* (Ezaki *et al.*, 2000). Expression of a wheat Mn-superoxide dismutase also conferred increased Al resistance in transgenic canola (Basu *et al.*, 2001).

Several gene products have been shown to be Al-induced by analysis of their activity. These include glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Slaski *et al.*, 1996) and vacuolar H^+-ATPase (Kasai *et al.*, 1992, 1993). In contrast, the plasma membrane H^+-ATPase (Matsumoto, 1988; Sasaki *et al.*, 1995; Widell *et al.*, 1994) is inhibited by Al. Glucan synthase II is inhibited by Al *in vitro* (Widell *et al.*, 1994), but synthesis of its end-product, callose, is stimulated by Al stress *in vivo* (Zhang *et al.*, 1994).

Another group of gene products have been shown to be Al-induced by comparing protein profiles of Al-sensitive and Al-resistant cultivars during Al stress (Ownby and Hruschka, 1991; Picton *et al.*, 1991; Delhaize *et al.*, 1991; Cruz-Ortega and Ownby, 1993; Somers *et al.*, 1996). Only three Al-induced proteins have been shown to co-segregate with the Al-resistance phenotype in wheat. One of these is a 23 kD root exudate protein (Basu *et al.*, 1997), while the other two are 51 kD, tonoplast associated proteins (RMP51), which are the focus of this study. These 51 kD proteins are specifically induced by Al in root tips of an Al-resistant cultivar of wheat (PT741). They are newly synthesized once Al stress begins, accumulate in a dose- and time-dependent manner, and then decline to control levels with the removal of Al stress (Basu *et al.*, 1994). The identity of these proteins remained to be determined.

The aim of the present study was to identify and characterize the RMP51 proteins in order to better understand their role in the Al stress response. These proteins have been purified from an endomembrane-enriched membrane fraction isolated from root tips of an Al-resistant cultivar of *Triticum aestivum* L. (cv PT741) grown in the presence of Al. Their identity has also been determined using peptide microsequence data. Purified peptides were homologous to the B subunit of the V-ATPase and to the α and β subunits of F_1F_0-ATPase (mitochondrial ATP synthase). Both V-ATPase and F_1F_0-ATPase activities were induced by Al treatment in PT741 and not in other cultivars tested. These results suggest that upregulation of ATPase activity in PT741 may be an adaptive response involved in Al resistance.

2.2 Methods

2.2.1 *Plant Material and Growth Conditions*

Seeds of Al-resistant (PT741, Atlas-66, Maringa) and Al-sensitive (Scout-66, Katepwa) cultivars of *Triticum aestivum* L. (wheat) were surface sterilized in 0.5% (v/v) sodium hypochlorite for 15-20 min and germinated overnight in a 0.005 g L^{-1} solution of the antifungal agent Vitavax (Uniroyal Chemical Ltd, Calgary, AB, Canada) in double distilled water to limit fungal growth. Seedlings were grown for 5 d on nylon mesh floating over 15 L of an aerated mineral nutrient solution (pH 4.30) containing (μM): 2900 NO_3, 300 NH_4, 100 PO_4, 800 K, 1000 Ca, 300 Mg, 101 SO_4, 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, 0.1 Mo and 10 EDTA. For Al exposure, 5 d old seedlings were transferred to solutions containing (μM): 1000 Ca, 300 Mg, 300 NH_4, 2900 NO_3 and 0-100 $AlCl_3$ at pH 4.30 (Taylor *et al.*., 1997). The seedlings were grown in a growth chamber (16 h light, 20°C, 68% RH and 8 h darkness, 16°C, 85% RH) for 2 d. Taylor *et al.* (1997) demonstrated that 100 μM $AlCl_3$ is optimal for induction of the proteins of interest in cv. PT741. After 2 d of Al exposure, 1 cm root tips were harvested for isolation of endomembranes and subsequent purification of the 51 kD proteins (RMP51).

2.2.2 *Isolation of Endomembrane-Enriched Membranes*

All steps involved in endomembrane preparation were carried out at 4°C. Root tips (1 cm) were finely chopped and immediately homogenized in a Proctor-

Silex blender in homogenization buffer (0.25 M sucrose, 50 mM MOPS-Tris pH 7.5, 5 mM EDTA and 5 mM ascorbic acid), 1 mL per g root tissue. The homogenate was then filtered through miracloth (Calbiochem) and centrifuged at 20,000g for 15 min. The supernatant was collected and centrifuged at 100,000g for 1 h. The microsomal membrane pellet was resuspended in gradient buffer (0.25 M sorbitol, 5 mM HEPES-BTP, pH 7.0) and loaded onto a two-step gradient of 5 mL each of 2% and 10% (w/w) Dextran T-70 prepared in gradient buffer (Kasai *et al.*, 1992). The gradient was centrifuged at 70,000g for 2 h, and the interface containing endomembranes was collected, diluted with gradient buffer, and centrifuged at 120,000g for 1 h. The endomembrane-enriched membrane pellet was resuspended in 10 mM Tris-Acetate, pH 7.9, 10% glycerol and either frozen at -80°C for marker enzyme analysis, or used immediately for further purification.

2.2.3 Membrane Marker Assays

To ensure that the isolated membrane fraction was enriched for endomembranes, marker enzyme analysis was carried out according to Briskin *et al.* (1987). Activities of the NO_3^--and bafilomycin-sensitive ATPases, vanadate-sensitive ATPase and glucan synthase II (GSII), and cytochrome c oxidase were used as markers for tonoplast, plasma membrane and mitochondria, respectively. Adenosine triphosphatase activity was assayed in a reaction mixture containing 30 mM Tris-MES (pH 8.0), 3 mM $MgSO_4$, 0.2% Triton X-100 (v/v), 50 mM KCl, and 3 mM ATP-Tris, in the presence or absence of 250 µM Na_3VO_4 (P-Type ATPase activity), 1 mM NaN_3 (F_1F_0-Type ATPase activity), 100 nM bafilomycin A_1 or 50 mM KNO_3 (V-

Type ATPase activity). Phosphatase activity was measured as described by Ames (1966).

2.2.4 *Purification of RMP51*

Separation of the 51 kD proteins (RMP51) from other endomembrane-associated proteins was achieved by continuous elution electrophoresis (Miniprep Cell: Bio-Rad Laboratories). Total endomembrane protein (up to 500 µg per run), was prepared for electrophoresis by adding an equivalent volume of SDS-PAGE loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue) and heating at 95°C for 5 min. Separating conditions for SDS-PAGE were as follows: 1.5 cm (4%) stacking gel, 10 cm (10%) separating gel. Electrophoresis/elution was carried out at 4°C at a constant current of 5 mA. Fractions (200 µL) were collected after the dye front had run off the gel and analyzed for the presence of RMP51 by SDS-PAGE (Mini Protean II: Bio-Rad Laboratories). Fractions containing RMP51 from each run were pooled and desalted using Sephadex G-50 gel filtration chromatography (Nick Column: Amersham Pharmacia Biotech). Aliquots of desalted samples were then analyzed for purity using two-dimensional electrophoresis (Mini Protean II 2D Cell: Bio-Rad Laboratories) according to the manufacturer's directions. After confirming their purity by two-dimensional electrophoresis, the desalted fractions from each run were pooled for microsequence analysis.

2.2.5 *SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Samples were prepared for SDS-PAGE by adding an equivalent volume of SDS-PAGE loading buffer and heating at 95°C for 5 min. Running conditions for SDS-PAGE (Laemmli, 1970) were: 4% stacking gel (at 12.5 mA) and 10% resolving gel (at 25 mA).

2.2.6 *Two-Dimensional Electrophoresis*

First dimension isoelectric focusing gels [4% acrylamide, 9.2 M urea, 2.0% CHAPS, 1.6% 5/7 ampholyte (BioRad Laboratories), and 0.4% 3/10 ampholyte (BioRad Laboratories)] were prefocused at 200V/10 min, 300V/15 min, and 400V/15 min. Microsomal membrane samples were extracted and precipitated as described by Hurkman and Tanaka (1986). Approximately 250 ng purified protein or 20 µg crude protein was then mixed with IEF sample buffer [9.5 M urea, 2.0% CHAPS, 5% β-mercaptoethanol, 1.6% 5/7 ampholyte (BioRad Laboratories), 0.4% 3/10 ampholyte (BioRad Laboratories)] and heated at 70°C for 20 min. After changing electrolytes and sample loading, isoelectric focusing was run for 500V/10 min and 750V/3.5h using the Mini Protean II 2D Cell (Bio-Rad Laboratories). Second dimension electrophoresis was carried out as described above.

2.2.7 *Protein Quantitation and Visualization*

Quantitation of protein present in membrane samples was performed using either the Bradford assay (Bradford, 1970) or comparing the band density of samples and known quantities of a standard protein (chicken egg ovalbumin: Sigma A5503)

of similar molecular weight (45 kD) on denaturing polyacrylamide gels. Proteins present in polyacrylamide gels were visualized using either a modified Morrissey silver stain procedure (Morrissey, 1981; Merril *et al.*, 1981) or staining with Coomassie Brilliant Blue R-250 (CBB R-250 Staining Kit, Bio-Rad Laboratories).

2.2.8 *Immunoblotting*

Microsomal proteins and RMP51 were separated by SDS-PAGE and electroblotted onto nitrocellulose (0.45µm, BioRad Laboratories) membranes using the Mini Trans-Blot Cell (Bio-Rad Laboratories) with a transfer buffer of 25 mM Tris, 192 mM glycine (pH 8.3) and 20% methanol. Transfer was performed at 100V at 4^0C for 1h. Membranes were blocked overnight in TBST (20 mM Tris, pH 7.5, 140 mM NaCl, 0.1% v/v TWEEN-20) and 5% w/v skim milk powder. Membranes were incubated with primary antibodies diluted in TBST at 37^0C for 1h, followed by three 15 min washes in PBST (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% TWEEN-20). Membranes labeled with primary antibody were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma A6154) diluted 1:15, 000 in PBST at 4^0C for 1h, followed by three 15 min washes. Chemiluminescent detection (Kirkegaard and Perry Laboratories, 54-61-00) was carried out according to the manufacturer's directions.

2.2.9 *Peptide Microsequence Analysis*

A 10 µg sample of purified RMP51 was electrophoresed and excised from a denaturing SDS-polyacrylamide gel and sent to the Harvard Microchemistry Facility

(Cambridge, MA) for tryptic digestion, peptide separation and microsequence analysis. With technical advice from Harvard Microchemistry, the most abundant tryptic peptides were selected for microsequence analysis. Sequence comparison of the selected peptides was performed using the BLAST algorithm (Altschul *et al.*, 1997).

2.2.10 RNA Isolation, Northern Hybridization and Analysis

RNA was isolated from 1 cm root tips using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's directions. Each 100 mg tissue sample yielded approximately 50 µg of RNA. RNA concentration was estimated by measuring A_{260} and checked by running aliquots on non-denaturing 1% agarose gels. Size Standards (Gibco-BRL RNA ladder) were included on all gels. Northern transfers were carried out using GeneScreen Plus (DuPont) membranes according to the manufacturer's directions. Probes for Northern blots were prepared from cDNA fragments isolated from pHTB1 (Berkefman *et al.*, 1994) and pAH (Chaumont *et al.*, 1988) plasmids (V-ATPase B subunit from *Hordeum vulgare* and F_1F_0-ATPase α subunit from *Nicotiana plumbaginafolia*, respectively). Plasmids were digested with appropriate restriction enzymes (Amersham Pharmacia Biotech) and the released cDNA inserts were isolated from agarose gels using the QiaQuick gel extraction kit (Qiagen). Probes were then prepared by random priming (Sambrook *et al.*, 1989) using 100 ng of each DNA template. Unincorporated nucleotides (including [^{32}P]dCTP) were removed using Sephadex G-50 size exclusion chromatography (Nick Column: Amersham Pharmacia Biotech). Membranes were prehybridized at

42^0C with 100 µg ml^{-1} denatured, sheared, herring sperm DNA (Sigma) for 4h. Hybridization was carried out overnight at 42^0C and washed twice with 2X SSPE (1X SSPE: 0.15M NaCl, 0.01 M NaH_2PO_4-H_2O, 0.001 M EDTA-Na_2, pH 7.4) for 15 min at room temperature, twice with 2X SSPE, 2% SDS for 45 min at 65^0C, and twice with 0.1X SSPE for 15 min at room temperature. Membranes were exposed to Kodak X-OMAT x-ray film at -80^0C. Shorter exposures of autoradiograms were performed to confirm that the observed results were not due to film saturation. Transcript levels were measured by densitometry of autoradiographs using an Alphaimager 2000 Documentation and Analysis System (Version 5.1 Alpha Innotech Corp). For Fig preparation, representative lanes of triplicate samples were selected from the same blot and compiled using Adobe Photoshop^R version 5.5.

2.3 Results

2.3.1 Purification of RMP51

The first step in the purification of RMP51 was preparation of endomembrane-enriched membranes from Al-treated seedlings of *Triticum aestivum* L. cv PT741. Membrane enrichment was confirmed using marker enzyme analysis (Briskin *et al.*, 1987). Positive markers for the tonoplast (Bafilomycin A_1- and nitrate-sensitive ATPase activities) were enriched by 1.43x and 3.35x, respectively, while negative markers (vanadate-sensitive ATPase, glucan synthase II and cytochrome c oxidase activities) were reduced to 0.45x, 0.69x and 0.35x (Table 2-1). Other endomembrane-associated proteins were then separated from RMP51 by

Table 2-1. Enrichment of marker enzymes in endomembrane-enriched membrane fractions isolated from root tips of *Triticum aestivum* L. cv PT741. Endomembrane-enriched membranes were isolated from microsomal membranes using a two-step gradient of Dextran T70 (2% and 10%), from which the interface was collected. Data are representative of 3 independent membrane preparations. Values shown are means +/- SE.

Marker Enzyme	Cellular Location	Enrichment in Endomembrane Fraction*	
		Mean	SE
Bafilomycin A_1-Sensitive ATPase	Tonoplast	1.43	0.24
Nitrate-Sensitive ATPase	Tonoplast	3.35	1.15
Vanadate-Sensitive ATPase	Plasma Membrane	0.45	0.19
Glucan Synthase II	Plasma Membrane	0.69	0.21
Cytochrome C Oxidase	Mitochondria	0.35	0.02

*Enrichment in endomembrane fraction is calculated as endomembrane specific activity/microsomal specific activity

continuous elution electrophoresis using the Miniprep Cell (BioRad). Separating conditions were optimized to purify the RMP51 band in a single step (Fig 2-1). The optimized procedure was repeated five times, with the RMP51 proteins isolated as a single band each time.

To determine whether or not both RMP51 proteins were present in the purified 51 kD band, aliquots of the pooled fractions from each purification run were analyzed by two-dimensional electrophoresis. Although two RMP51 spots were visible in the crude endomembrane fraction (Fig 2-2A), only one spot was visible in the purified sample (Fig 2-2B). This may have been caused by the presence of residual SDS (even after detergent removal and addition of nonionic CHAPS) or by loss of one of the RMP51 proteins during purification. Only one protein spot was observed from every preparation. Since the results from each purification run were identical, all of the fractions containing RMP51 were pooled. Total protein yield from five independent preparations was approximately 15 µg, sufficient for subsequent analysis by tryptic digestion and peptide microsequencing.

2.3.2 Peptide Microsequence Analysis

Tryptic digestion and fragment purification by HPLC were carried out at the Harvard Microchemistry Facility (Cambridge, MA). Expert advice from Harvard helped to select several peptides which were abundant and well-separated from other

Fig 2-1. Fractionation of endomembrane-associated proteins by continuous elution electrophoresis. Total endomembrane proteins were isolated from root tips of *Triticum aestivum* L. cv PT741 after 48h treatment with 100 µM $AlCl_3$. Selected fractions (lanes 2-8) were analyzed for the presence of RMP51 (arrow) by SDS-PAGE and silver staining. Molecular weights of protein standards are given in kilodaltons. Fractions pooled for further analysis are indicated by asterisks (*).

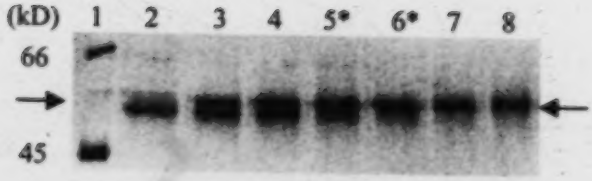
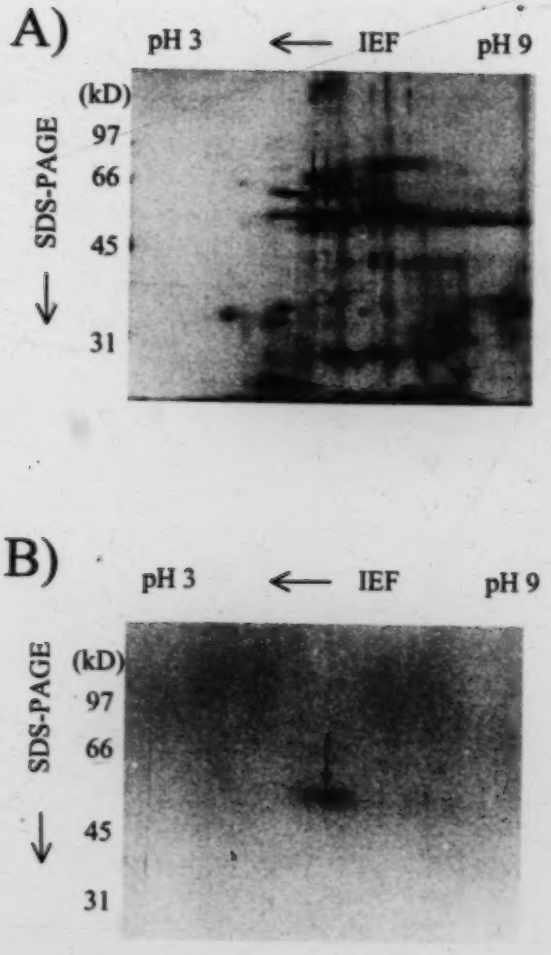

(kD)
1
2
3
4
5*
6*
7
8
66
45

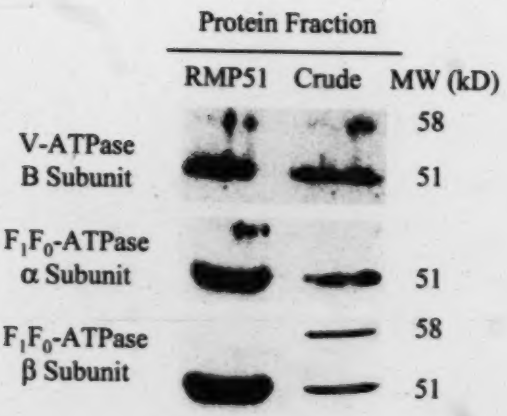
Fig 2-2. Two-dimensional gel analysis of crude endomembrane proteins (A) and purified RMP51 (B). Molecular weights of protein standards (kD) are indicated on the left and the pH scale is shown on the top. RMP51 is indicated by solid arrows. Data shown are representative of 5 independent trials.


A)
pH 3
IEF
pH 9
(kD)
97
66
45
31
SDS-PAGE
B)
pH 3
IEF
pH 9
(kD)
97
66
45
31
SDS-PAGE

peptides by HPLC. Four of these peptides were selected, sequenced and subsequently identified using the BLAST sequence algorithm (Altschul *et al.*, 1997). Peptide 1 (QIYPPINVLPSLSR) was identical to amino acids 365-378 of the V-ATPase B subunit from barley (*Hordeum vulgare*, accession Q40079). Peptide 2 (FVAQGAYDTR) also showed 100% identity to the barley V-ATPase B subunit (accession Q40079, amino acids 440-449). Peptide 3 (FTAQANSEVSALLGR) showed 100% identity to amino acids 336-349 of the F_1F_0-ATPase β subunit from wheat (accession P20858), but no significant homology to the V-ATPase B subunit. Peptide 4 (TGSIVDVPAGK) showed 100% identity to amino acids 93-103 of the F_1F_0-ATPase α subunit from wheat (accession P12862).

Results from the BLAST sequence comparison suggest that the four tryptic fragments were derived from the ATP-binding and catalytic subunits of related ATPases (V-ATPase and F_1F_0-ATPase). The possibility that they are all derived from a single protein was checked by aligning the four RMP51 peptide sequences with the B subunit of the V-ATPase from barley and the α and β subunits of the F_1F_0-ATPase from wheat. The 3 ATPase subunits share only approximately 25% identity, and the peptide sequences are not derived from regions with significant sequence homology between them. Therefore, it is unlikely that any single protein could contain all four peptides. The fact that more than one protein is present in the RMP51 band was confirmed by Western analysis (Fig 2-3). Antibodies raised against the V-ATPase B subunit from *Vigna radiata* and the F_1F_0-ATPase α and β subunits from

Fig 2-3. Identification of RMP51 by immunoblotting. RMP51 and crude microsomal proteins isolated from Al-treated root tips of *Triticum aestivum* L. cv PT741 were separated by SDS-PAGE, immunoblotted and probed with polyclonal antibodies specific to the V-ATPase B subunit (from *Vigna radiata*) and the F_1F_0-ATPase α and β subunits (from *Saccharomyces cerevisiae*). Antibody labeling was detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent detection.

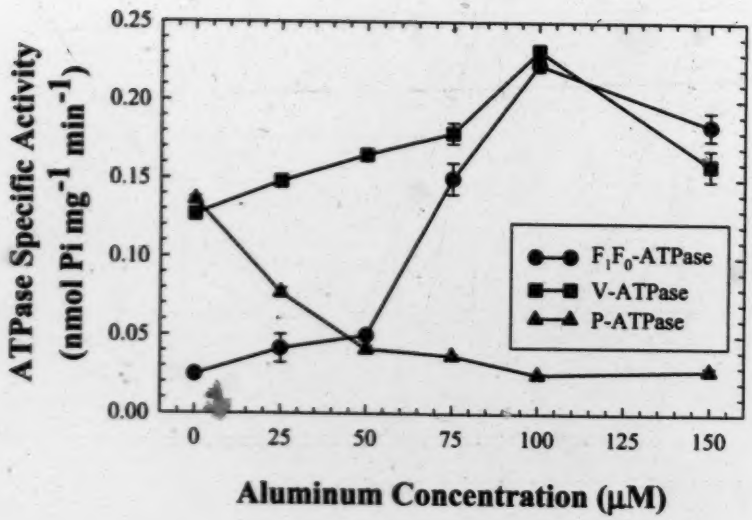

Protein Fraction
RMP51
Crude
MW (kD)
V-ATPase
B Subunit
58
51
F_1F_0-ATPase
α Subunit
51
F_1F_0-ATPase
β Subunit
58
51

Saccharomyces cerevisiae all cross-react with RMP51, suggesting that all three proteins are present.

2.3.3 V-ATPase and F_1F_0-ATPase are Al-induced

Since RMP51 protein levels increase in a dose-dependent manner with Al treatment (Taylor *et al.*, 1997), levels of V-ATPase and F_1F_0-ATPase activity in Al-treated and control seedlings were compared. Activities were measured in total microsomal membrane fractions isolated from 5d-old seedlings of *Triticum aestivum* L. cv PT741 exposed to 0-150 μM $AlCl_3$ for 2d (Fig 2-4). V-ATPase and F_1F_0-ATPase activities were both induced during Al stress, although to different extents. Vacuolar ATPase activity increased to 181% of control (from 0.127 to 0.232 nmol Pi mg^{-1} min^{-1}) as concentrations of $AlCl_3$ increased from 0-100 μM, followed by a decline to 125% of the control level (0.159 nmol Pi mg^{-1} min^{-1}) at 150 μM. Mitochondrial F_1F_0-ATPase activity increased to 899% of the control level (from 0.025 to 0.223 nmol Pi mg^{-1} min^{-1}) as concentrations of $AlCl_3$ increased from 0-100 μM, followed by a decline to 742% of the control level (0.184 nmol Pi mg^{-1} min^{-1}) at 150 μM $AlCl_3$. Plasma membrane ATPase activity decreased to 57% of the control level (from 0.136 to 0.077 nmol Pi mg^{-1} min^{-1}) at 25 μM $AlCl_3$ and to 18% of the control level (0.025 nmol Pi mg^{-1} min^{-1}) at 100 μM. Since both F_1F_0-ATPase and V-ATPase activities were induced by Al in a dose-dependent manner, it remains possible that the Al-induced RMP51 band initially identified by Basu *et al.* (1994a) consisted of both vacuolar and mitochondrial components.

Fig 2-4. The effect of Al on activity of the V-ATPase, F_1F_0-ATPase and P-ATPase. ATPase specific activity was measured in total microsomal membranes prepared from 1 cm root tips of the Al-resistant cultivar, PT741. Five-d-old seedlings were treated with different concentrations of Al (0, 25 50, 75, 100, 150 μM) for 48h. Values are means +/- SE of three biological replicates and are representative of three independent trials.


ATPase Specific Activity
(nmol Pi mg-1 min-1)
0.25
0.20
0.15
0.10
0.05
0.00
0
25
50
75
100
125
150
Aluminum Concentration (μM)
F1F0-ATPase
V-ATPase
P-ATPase

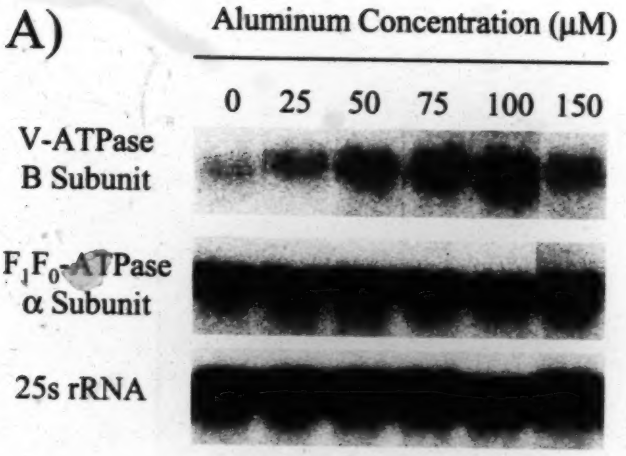
In order to determine whether or not there is a transcriptional component to the induction of these ATPases, Northern analysis was performed on RNA isolated from the same tissues used for ATPase assays (Fig 2-5). Levels of the transcript encoding the B subunit of the V-ATPase showed an increase to 1.9x control from 0-100 μM $AlCl_3$. This increase, although relatively small, was consistent in 3 independent experiments, and showed a pattern similar to that observed for V-ATPase activity (Fig 2-4). This suggests that induction of V-ATPase activity by Al may be transcriptionally mediated, although participation of translational and post-translational mechanisms cannot be excluded. In contrast, levels of the transcript encoding the α subunit of the F_1F_0-ATPase remained constant over the entire range of Al concentrations tested. Since F_1F_0-ATPase activity levels increased 899% over this range, it appears that this increase may be due to a translational or post-translational mechanism.

2.3.4 Cultivar Screen for ATPase Induction

If induction of V-ATPase and F_1F_0-ATPase activities are general responses to Al stress, then induction should be observed in Al-resistant and Al-sensitive cultivars of wheat under stress conditions. To test this hypothesis, V-ATPase, F_1F_0-ATPase and P-ATPase activities were measured in 3 Al-resistant and 2 Al-sensitive cultivars of wheat in control (0 μM $AlCl_3$) conditions, conditions stressful to sensitive cultivars (20 μM $AlCl_3$), and conditions stressful to resistant cultivars (100 μM $AlCl_3$) (Taylor

Fig 2-5. The effect of Al on transcript levels of V-ATPase and F_1F_0-ATPase subunits. RNA was isolated from the same tissues used for ATPase activity measurements in Fig 4 and Northern blotted. (A) Autoradiographs of Northern blots probed with ^{32}-P labeled cDNAs encoding the V-ATPase B subunit from *Hordeum vulgare* and the F_1F_0-ATPase α subunit from *Nicotiana plumbaginafolia*. To ensure equal RNA loading and transfer, membranes were also probed with a cDNA clone encoding the 25s rRNA from *Glycine max*. Results shown are representative of three independent trials. (B) Quantification of transcript levels shown in (A). Transcript levels are expressed as the density of each band relative to 25S rRNA on the autoradiograph. Values are means +/- SE of three independent replicates.

A)


Aluminum Concentration (μM)
0 25 50 75 100 150
V-ATPase
B Subunit
F_1F_0-ATPase
α Subunit
25s rRNA

B)

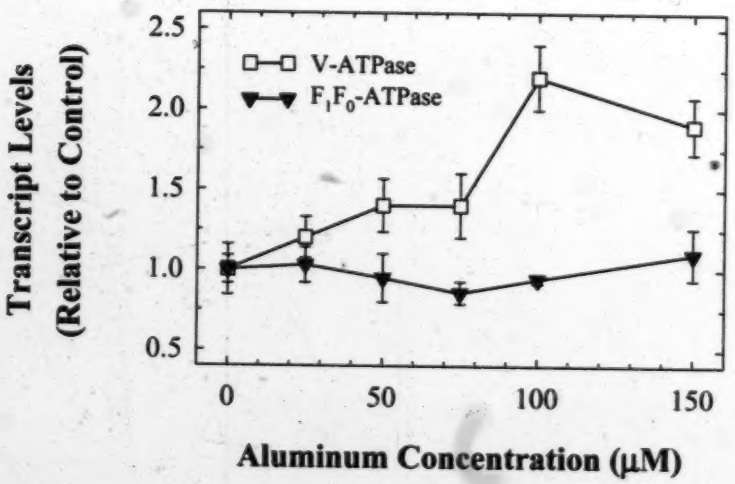
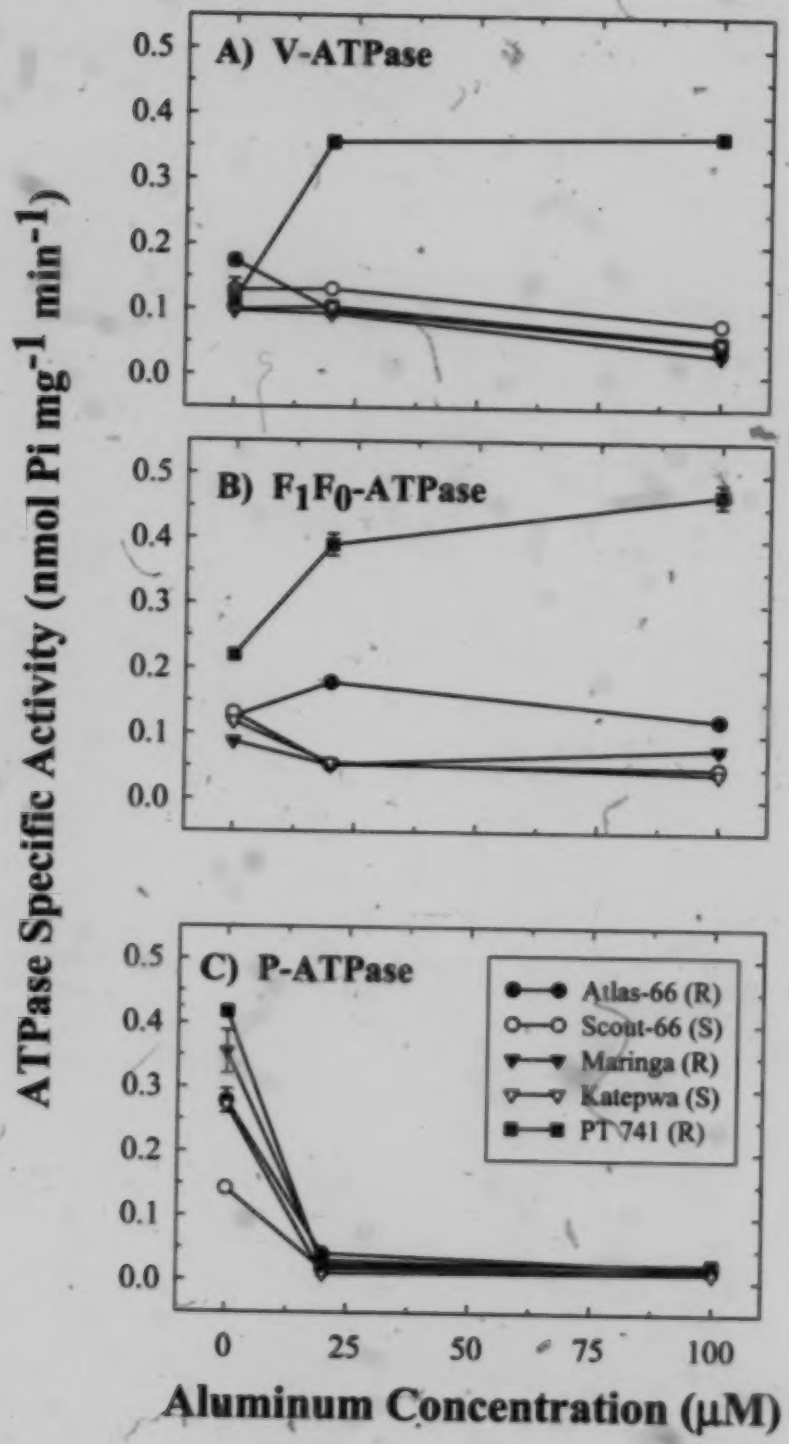

Transcript Levels
(Relative to Control)
2.5
2.0
1.5
1.0
0.5
V-ATPase
F_1F_0-ATPase
0
50
100
150
Aluminum Concentration (μM)

Fig 2-6. The effect of Al on ATPase specific activity in total microsomal membranes prepared from 1 cm root tips of PT741, Atlas-66 and Maringa (Al-resistant), and from Scout-66 and Katepwa (Al-sensitive). Five-d-old seedlings of each cultivar were exposed to 0, 20 or 100 μM Al for 48h. Values represent means +/- SE of three independent replicates.


A) V-ATPase
B) F_1F_0-ATPase
C) P-ATPase
ATPase Specific Activity (nmol Pi mg^{-1} min^{-1})
Aluminum Concentration (μM)
0.0
0.1
0.2
0.3
0.4
0.5
0
25
50
75
100
Atlas-66 (R)
Scout-66 (S)
Maringa (R)
Katepwa (S)
PT 741 (R)

et al., 1997). In cultivar PT741, V-ATPase activity increased by 315% (from 0.113 to 0.356 nmol Pi mg^{-1} min^{-1}), even at 20 µM $AlCl_3$ (Fig 2-6A). Vacuolar ATPase activities in Atlas-66 and Maringa (Al-resistant) were unaffected at 20 µM $AlCl_3$, but reduced to 30% and 33% of control (from 0.174 to 0.050 nmol Pi mg^{-1} min^{-1} and from 0.097 to 0.032 nmol Pi mg^{-1} min^{-1}, respectively) at 100 µM $AlCl_3$. In Scout-66 and Katepwa (Al-sensitive), a similar pattern was observed, with reductions to 62% and 54% of control (from 0.129 to 0.080 nmol Pi mg^{-1} min^{-1} and from 0.097 to 0.032 nmol Pi mg^{-1} min^{-1}, respectively) observed at 100 µM $AlCl_3$.

Cultivar PT741 also showed induction of F_1F_0-ATPase activity in this experiment (Fig 2-6B). Control levels of F_1F_0-ATPase activity were slightly greater in PT741 than in the other cultivars tested, and activity increased to 180% of control (from 0.220 to 0.390 nmol Pi mg^{-1} min^{-1}) at 20 µM $AlCl_3$ and to 214% of control (0.469 nmol Pi mg^{-1} min^{-1}) at 100 µM $AlCl_3$. In Atlas-66, F_1F_0-ATPase activity increased to 142% of control (from 0.125 to 0.178 nmol Pi mg^{-1} min^{-1}) at 20 µM $AlCl_3$, but activity declined to control levels at 100 µM $AlCl_3$. All other cultivars tested showed reduced F_1F_0-ATPase activities (Maringa, Scout-66 and Katepwa reduced to 59%, 40% and 47% of control, respectively) at 20 µM $AlCl_3$. In contrast to the above observations, decreased activity of the plasma membrane ATPase was a general effect of Al treatment in all cultivars tested (Fig 2-6C). Activities decreased to near zero in all cultivars, even at 20 µM $AlCl_3$. In summary, V-ATPase and F_1F_0-

ATPase activities were specifically induced in PT741, while P-ATPase activity declined in all cultivars tested.

2.4 Discussion

Differential screening of mRNA and protein profiles between Al-treated and untreated tissues or between Al-resistant and Al-sensitive wheat cultivars has revealed that expression of many genes is induced during Al stress (Snowden and Gardner, 1993; Snowden *et al.*, 1995; Richards *et al.*, 1994; Cruz-Ortega *et al.*, 1997; Hamel *et al.*, 1998; Basu *et al.*, 2001). However, only three Al-induced proteins (Basu *et al.*, 1994b; Taylor *et al.*, 1997) have been shown to co-segregate with the Al-resistance phenotype. In order to better understand the role of these proteins, their identities must be determined.

Aluminum stress causes a general decline in protein synthesis (^{35}S-methionine incorporation) in Al-sensitive cultivars of *Triticum aestivum*, while there is little to no effect in Al-resistant cultivars (Ownby and Hruschka, 1991; Rincon and Gonzales, 1991; Basu *et al.*, 1994a). Similar results have been observed in *Medicago sativa* (Campbell *et al.*, 1994). Since pretreatment of Al-resistant cultivars of wheat (Atlas-66, Grana) improves the resistance of plants to subsequent exposures, Aniol (1984) hypothesized that induction of new protein synthesis was involved in Al resistance. This hypothesis was supported by the subsequent observation that Al pretreatment induced Al tolerance in an Al-resistant cultiwar of *Phaseolus vulgaris* L. (Cumming *et*

al., 1992). Basu *et al.* (1994a) previously reported that two tonoplast-associated proteins are induced by Al stress in root tips of an Al-resistant cultivar of *Triticum aestivum*, PT741. These proteins segregate with the Al-resistance phenotype in a cross between PT741 and Katepwa (Taylor *et al.*, 1997). In this work, I used continuous elution electrophoresis to purify the 51 kD band containing these proteins from Al-treated seedlings of *Triticum aestivum* L. cv PT741 (Fig 2-1). Sequence analysis of purified peptides was then used to identify RMP51. Of four peptides sequenced, two were identical to fragments of the V-ATPase B subunit from barley, one was identical to a fragment of the F_1F_0-ATPase α subunit from wheat and one was identical to a fragment of the F_1F_0-ATPase β subunit from wheat. Although RMP51 was originally identified as two proteins, it was nonetheless surprising that both mitochondrial and vacuolar proteins were present in the 51 kD band, which had been purified from an endomembrane-enriched fraction (Table 2-1). However, antibodies specific to the B subunit of the V-ATPase and the α and β subunits of the F_1F_0-ATPase all cross-reacted with the RMP51 band (Fig 2-3), suggesting that there were in fact at least three proteins present in the purified band.

To determine whether the V-ATPase and the F_1F_0-ATPase are Al-induced, ATPase activities were measured at various Al concentrations. Basu *et al.* (1994a) showed previously that RMP51 protein levels increased in an Al dose-dependent manner. If RMP51 is in fact a subunit of the V-ATPase or F_1F_0-ATPase, then activities of these enzymes should show a similar response to Al, assuming that all of the subunits are similarly induced. My data showed that V-ATPase and F_1F_0-ATPase

activities increased to 183% and 899% of control (respectively) with Al treatment, while P-ATPase activity decreased to 18% of control levels (Fig 2-4). Induction of V-ATPase (and H^+-PP_iase) by Al has been previously demonstrated in tonoplast-enriched membrane vesicles from *Hordeum vulgare* roots by Kasai *et al.* (1992, 1993). These authors observed a 40-53% increase in V-ATPase activity upon Al exposure, depending upon the external Ca^{2+} concentration. These results were confirmed by Zhang *et al.* (1998). Inhibition of P-ATPase activity has also been previously reported. Matsumoto (1988) observed a 50% decrease in P-ATPase activity from *Hordeum vulgare* roots treated with 100 μM $AlCl_3$ at pH 6.5, and a 45% decrease after treatment with 1 mM $AlCl_3$ at pH 5.5 (Matsumoto *et al.*, 1992). In subsequent experiments, Sasaki *et al.* (1995) showed that P-ATPase activity in *Triticum aestivum* declined by 13-19% after treatment with 50 μM $AlCl_3$ at pH 4.5. Widell *et al.* (1994) observed a similar effect in *Picea abies* and *Triticum aestivum*.

The effect of Al treatment on F_1F_0-ATPase activity has not previously been investigated. I was initially surprised by the induction of F_1F_0-ATPase activity, since localization experiments showed no enrichment of the RMP51 band in mitochondrial fractions (Taylor *et al.*, 1997). However, it is possible that protein levels of the mitochondrial F_1F_0-ATPase subunits remain constant during Al stress, and that F_1F_0-ATPase activity is being modulated at the post-translational level (Stevens and Forgac, 1997). This is consistent with our observation that steady-state transcript levels remained constant while activity increased by 7.3x (Figs 2-4, 2-5). Since Al induces F_1F_0-ATPase activity, increased ATP synthase activity may be required to

support V-ATPase induction and other energy-dependent processes involved in Al resistance. Although ATP synthase activity was not directly measured, the F_1F_0-ATPase acts almost exclusively as an ATP synthase under physiological conditions. Therefore, the increased F_1F_0-ATPase activity observed here has been interpreted as increased ATP synthase activity *in vivo*.

If induction of V-ATPase and F_1F_0-ATPase activities is a general effect of Al stress, then a similar response should be observed in all cultivars, whether Al-resistant or Al-sensitive. If induction is involved in mediating a general Al-resistance mechanism, induction should occur only in Al-resistant cultivars. Alternatively, induction may be a cultivar-specific resistance mechanism in PT741. To differentiate among these hypotheses, ATPase activities were measured in two additional Al-resistant cultivars (Atlas-66, Maringa) and two Al-sensitive cultivars (Scout-66, Katepwa). Our data show that Al-induction of V-ATPase and F_1F_0-ATPase is unique to PT741 (Fig 2-6). In all other cultivars, V-ATPase and F_1F_0-ATPase activities remained constant or declined with Al treatment.

It is interesting that this phenomenon was observed only in PT741 and not in the other two Al-resistant cultivars tested, neither of which showed increased levels of RMP51 protein (data not shown). The lack of an effect in Maringa is perhaps not surprising since research on this cultivar has correlated exudation of a 23 kD, Al-binding polypeptide with Al resistance, and Al-resistance is controlled by a single dominant gene (Basu *et al.*, 1997). In Atlas 66, Al resistance has also been correlated with enhanced exudation of malate during Al stress (Basu *et al.*, 1994c).

Several observations suggest that induction of V-ATPase and F_1F_0-ATPase could be involved in mediating Al resistance in *Triticum aestivum* L. cv PT741. First, RMP51 protein (consisting of the V-ATPase B subunit and the F_1F_0-ATPase α and β subunits) is newly synthesized upon Al treatment and accumulates in an Al dose-dependent manner (Basu *et al.*, 1994a). Second, accumulation of RMP51 segregates with the Al-resistance phenotype (Taylor *et al.*, 1997). Third, V-ATPase and F_1F_0-ATPase activities increase in an Al dose-dependent manner only in the Al-resistant cultivar, PT741.

Induction of V-ATPase activity has been linked to salt tolerance in several species (Ballesteros *et al.*, 1996; Kirsch *et al.*, 1996). Induction of V-ATPase activity is thought to be a homeostatic mechanism required to provide energy for Na^+/H^+ antiport, which delivers Na^+ to the vacuole (Matsumoto and Chung, 1988; Reuveni *et al.*, 1990; Nakamura *et al.*, 1992; Colombo and Cerana, 1993). Kasai *et al.* (1992) suggested that a similar mechanism could be responsible for Al resistance in wheat, with an Al^+/H^+ antiport system driving sequestration of Al in the vacuole. This suggestion should be taken with caution, however, since there is no direct evidence for an Al^+/H^+ co-transporter. Increased V-ATPase activity could also be required as a homeostatic mechanism to maintain the cytoplasmic pH near neutrality. I have shown that Al exposure decreases plasma membrane ATPase activity, in agreement with results reported previously (Matsumoto, 1988; Matsumoto *et al.*, 1992; Sasaki *et al.*, 1995; Widell *et al.*, 1994). This could cause a decrease in cytoplasmic pH, with adverse physiological effects. Increased activity of V-ATPase, with energy balance

maintained by increased ATP synthase activity, could counteract these changes by transporting protons into the vacuole. A similar phenomenon has been observed in *Lactobacillus acidophilus*, which expresses a pH-inducible F_1F_0-ATPase to extrude protons and maintain cytoplasmic pH (Kullen and Klaenhammer, 1999).

In summary, I have purified the Al-induced RMP51 proteins from the Al-resistant cultivar of wheat, PT741 and shown that the RMP51 band consists of the B subunit of the V-ATPase and the α and β subunits of the F_1F_0-ATPase. Vacuolar ATPase activity increased to 183-324% of control, while levels of the transcript encoding the B subunit increased by 2.2x, from 0-100 μM $AlCl_3$. Mitochondrial F_1F_0-ATPase activity increased by 214-899%, with a constant level of the transcript encoding the α subunit, over the same range of Al concentrations. Increased V-ATPase and F_1F_0-ATPase activities are observed only in PT741, suggesting that induction of V-ATPase and F_1F_0-ATPase activities is not simply symptomatic of stress. These results allow me to put forward the hypothesis that induction of the V-ATPase and the F_1F_0-ATPase plays a role in Al resistance in PT741. Moreover, the demonstration that tolerance to Al in PT741 is mediated by a different mechanism than is observed in other varieties of wheat (e.g. Maringa) suggests the possibility of combining these traits genetically to produce a cultivar with enhanced Al tolerance.

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3. Vacuolar H^+-ATPase, but not Mitochondrial F_1F_0-ATPase, is Required for Aluminum Resistance in *Saccharomyces cerevisae*[2]

3.1 Introduction

Although many mechanisms have been proposed to account for aluminum (Al) resistance in crop species (see for example Taylor, 1991), few have been tested directly. I have shown that vacuolar ATPase and mitochondrial F_1F_0-ATPase activities are induced by Al stress in the Al-resistant cultivar of wheat, PT741 (Chapter 2, Hamilton *et al.*, 2001). Synthesis of RMP51 (consisting of the B subunit of the V-ATPase and the α and β subunits of the F_1F_0-ATPase) is induced by Al in a dose- and time-dependent manner, and protein levels decline after removal of Al (Basu *et al.*, 1994). Aluminum-induced synthesis of RMP51 segregates with Al resistance in progeny derived from a cross between sensitive and resistant cultivars (Taylor *et al.*, 1997). This suggests that induction of V-ATPase and F_1F_0-ATPase may be an adaptive trait involved in Al resistance.

To further our understanding of Al resistance, I have developed a testable

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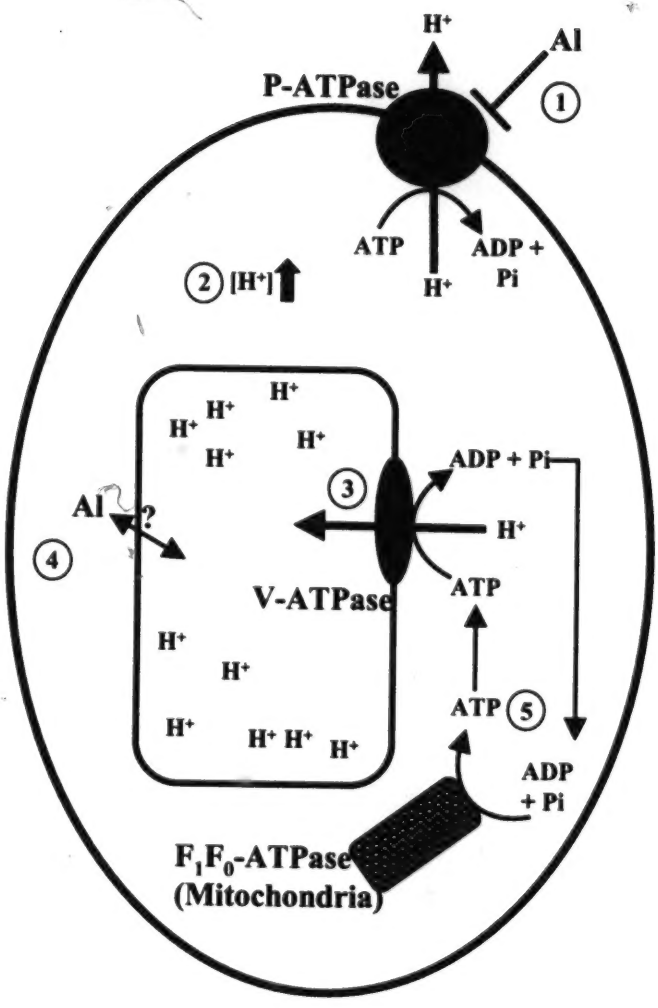
[2] A version of this chapter has been published. Hamilton CA, Good AG and Taylor GJ (2001) Vacuolar H^+-ATPase, but not Mitochondrial F_1F_0-ATPase, is Required for Aluminum Resistance in *Saccharomyces cerevisae*. FEMS Microbiol Lett **205**: 231-236

hypothesis describing the role of V-ATPase and F_1F_0-ATPase in Al resistance (Fig 3-1). Based on my model, inhibition of the plasma membrane ATPase (P-ATPase) by Al would cause proton accumulation in the cytoplasm and decreased cytoplasmic pH. To counteract this effect, I predict an increase in activity of the vacuolar ATPase (V-ATPase) which would transport excess protons into the lumen of the vacuole, thus alleviating the stress caused by cytoplasmic acidosis. Increased V-ATPase activity would also contribute to energizing the tonoplast membrane. It is possible that maintaining the tonoplast membrane electrochemical potential could provide energy required for a secondary active transport process involved in Al resistance. The ATP required for these processes would be provided by increased activity of ATP synthase (F_1F_0-ATPase). To test this model directly, I used yeast as a model system. Although yeast and plants do not necessarily respond to stress in a similar fashion, yeast has the advantages of rapid, simple transformation, short life cycle and amenibility to manipulation. This allows rapid hypothesis testing before, or while, more arduous experiments in plants are undertaken.

In this chapter I examined the response of wild type yeast to Al and tested the effect of mutations in V-ATPase and F_1F_0-ATPase subunits on Al resistance. If my model is correct, then inhibiting the activity of the V-ATPase should cause increased sensitivity to Al. Inhibiting the activity of the F_1F_0-ATPase may have a lesser effect on Al resistance, since yeast, unlike plants, can produce ATP to support V-ATPase activity via fermentation.

Fig 3-1. Model representing the energy balance hypothesis for V-ATPase and F_1F_0-ATPase-mediated Al resistance in wheat. (1) Inhibition of the plasma membrane ATPase (P-ATPase) by Al. (2) Proton accumulation in the cytoplasm causes cytoplasmic acidosis. (3) Increased activity of the vacuolar ATPase (V-ATPase) could transport excess protons into the lumen of the vacuole, alleviating stress caused by decreased cytoplasmic pH and energizing the tonoplast membrane. (4) It is possible that maintaining the tonoplast membrane potential could provide energy required for a secondary active transport process involved in a hypothetical Al resistance mechanism. (5) The energy required for these processes could be provided by increased activity of ATP synthase (F_1F_0-ATPase).


H+
Al
P-ATPase
1
ATP
ADP +
Pi
H+
2 [H+]
H+
H+
H+
H+
H+
ADP + Pi
3
Al
?
H+
4
ATP
V-ATPase
H+
H+
H+
H+ H+
H+
ATP
5
ADP
+ Pi
F1F0-ATPase
(Mitochondria)

3.2 Methods

3.2.1 Media and culture conditions

Yeast strains (Table 3-1) were maintained on standard media as described by Sherman (1991). For experiments using Al, a low pH, low phosphate (LPP) medium (Schott and Gardner, 1997) was used. This medium is synthetic complete (SC) minimal medium (0.67% yeast nitrogen base, 2% glucose) with K_2HPO_4 reduced to 100 µM, KCl added to 4.9 mM and the pH reduced to 3.5.

3.2.2 ATPase activity measurements

Seed cultures (3 ml in SC) of wild-type yeast were inoculated from YPD plate stocks and incubated for 16-24 h at 30°C. Cells were harvested by centrifugation, washed three times and resuspended in 1 ml of sterile water. This cell suspension was used to inoculate triplicate 3 ml cultures of LPP medium (pH 3.5) containing 0-15 µM $AlCl_3$ to a starting OD_{600} of approximately 0.01 and cultures were incubated for 16-24 h at 30°C (final OD_{600} approximately 0.9). Each 3 ml culture was harvested by centrifugation and spheroplasts prepared as described by Uchida *et al.* (1988). Cell suspensions were then sonicated at low intensity (Branson Sonifier, 10% output) for 30s to gently lyse cells. Cell debris was removed by centrifugation and the resulting supernatant used for enzyme assays. Quantitation of protein in cell extracts was performed using the Bradford assay (Bradford, 1976). Adenosine triphosphatase activity was then assayed as described by Briskin *et al.* (1987).

Table 3-1. Yeast strains used in this study

Strain	Genotype	Source
SF838-1D	*MATα, ade6, his4-519, leu2-3,112, ura3-52, pep4-3*	Rothman and Stevens, 1986
vma1	Isogenic to SF838-1D except *vma2Δ::LEU2*	Yamashiro *et al.*, 1990
vma2	Isogenic to SF838-1D except *vma1Δ::LEU2*	Kane *et al.*, 1990
vma1/VMA1	Isogenic to *vma1* except carrying wild-type *VMA1* on plasmid pRS316 (*URA3*)	This study
vma2/VMA2	Isogenic to *vma2* except carrying wild-type *VMA2* on plasmid pRS316 (*URA3*)	This study
W303-1A	*MATa, ade2-1, his3-1,15, leu2-3,112, trp1-1, ura3-1*	(Zhang *et al.*, 1999)
W303-Δα	Isogenic to W303-1A except null deletion mutation in *ATP1*	(Zhang *et al.*, 1999)
W303-Δβ	Isogenic to W303-1A except null deletion mutation in *ATP2*	(Zhang *et al.*, 1999)
W303-Δα/*ATP1*	Isogenic to W303-Δα except carrying wild-type *ATP1* on plasmid pRS314 (*TRP1*)	This study
W303-Δβ/*ATP2*	Isogenic to W303-Δβ except carrying wild-type *ATP2* on plasmid pRS316 (*URA3*)	This study

3.2.3 Complementation of ATPase mutations

Yeast strains carrying *LEU2* insertion mutations in *VMA1* and *VMA2*, as well as plasmids carrying the complementing genes, were provided by Dr Tom Stevens (University of Oregon). Strains carrying null deletion mutations of *ATP1* and *ATP2* and plasmids carrying the complementing genes were provided by Dr David Mueller (Chicago Medical School). In order to select for transformation of the plasmids in the appropriate mutant strains, *ATP1* and *VMA2* were recloned into pRS314 (*TRP1*) and pRS316 (*URA3*), respectively, using standard molecular biology techniques (Sambrook *et al.*, 1989). The complementing constructs were then transformed into appropriate yeast strains using the lithium acetate method (Geitz *et al.*, 1995).

3.2.4 Aluminum dose response experiments

Inoculum for each culture was prepared as described above and used to inoculate triplicate 3 ml cultures of SC medium containing 0-150 µM $AlCl_3$ to a starting OD_{600} of approximately 0.01. Cultures were incubated for 16-24 h at 30°C and the final OD_{600} of each culture was measured in a 96-well plate (Costar) using a Spectromax Plus plate reader and Softmax Pro v 1.1 software (Molecular Devices). For comparison, the OD_{600} values for each strain were expressed as a percentage of the control value for that strain.

3.2.5 Immunoblotting

Total yeast proteins were separated by SDS-PAGE (Laemmli, 1970) and electroblotted onto nitrocellulose (0.45 µm, BioRad Laboratories) membranes using the Mini Trans-Blot Cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked and probed as directed in the Lumi-glo Chemiluminescent Detection System manual (Kirkegaard and Perry Laboratories).

3.2.6 RNA Isolation, Northern Hybridization and Analysis

RNA was isolated from yeast cultures (approximately $5x10^7$ cells) using the RNeasy Kit (Qiagen) according to the manufacturer's directions. Northern transfers were carried out using GeneScreen Plus (DuPont) membranes according to the manufacturer's directions. Probes for Northern blots were isolated from pRS316VMA2 and pRS316ATP1 by digestion with appropriate restriction enzymes (Amersham Pharmacia Biotech) and inserts were isolated from agarose gels using the QiaQuick gel extraction kit (Qiagen). Probes were then prepared by random priming (Sambrook *et al.*, 1989). Membranes were prehybridized, hybridized and washed according to the manufacturer's directions, followed by exposure to storage phosphor screens (Molecular Dynamics). Phosphor screens were developed using a Phosphorimager 445SI and the resulting images analyzed using ImagequaNT v 4.2 software. For figure preparation, representative lanes of triplicate samples were selected from the same blot and compiled using Adobe Photoshop^R v 5.5.

3.3 Results

3.3.1 Response of yeast ATPases to aluminum

To confirm that yeast is a suitable model for investigating the effect of Al on ATPases in plants, the responses of ATPases to Al in wild type yeast were examined (Fig 3-2). F_1F_0-ATPase activity increased to 0.225 nmol Pi mg^{-1} min^{-1} (580% of control) at 2.5 μM $AlCl_3$. Although the Al-induced stimulation declined to 0.137 nmol Pi mg^{-1} min^{-1} (340% of control) from 5 to 10 μM $AlCl_3$, F_1F_0-ATPase activity was still 0.151 nmol Pi mg^{-1} min^{-1} (380% greater than control) at 15 μM, similar to what has been observed in wheat (Hamilton *et al.*, 2001). Although the magnitude of the change was less, P-ATPase activity showed an effect similar to what has been observed in plants (Hamilton *et al.*, 2001; Matsumoto, 1988; Matsumoto *et al.*, 1992; Sasaki *et al.*, 1995; Widell *et al.*, 1994), decreasing to 0.064 nmol Pi mg^{-1} min^{-1} (54% of control) at 15 μM Al. Vacuolar ATPase activity remained essentially constant from 0-10 μM $AlCl_3$. This is an interesting contrast to the Al-induced increase observed in wheat (Zhang *et al.*, 1998; Hamilton *et al.*, 2001) and barley (Kasai *et al.*, 1992, 1993).

To determine the mechanism by which ATPase activities change during Al stress, expression of V-ATPase and F_1F_0-ATPase subunits was examined by Western and Northern analysis (Fig 3-3). Levels of the transcript encoding the F_1F_0-ATPase α subunit increased to 201% of control levels by 7.5 μM $AlCl_3$, followed by a decline to 135% by 15 μM (Fig 3-3A,B). This pattern closely resembles that observed for increased F_1F_0-ATPase activity (Fig 3-2). Protein levels of the F_1F_0-ATPase α and β

Fig 3-2. The effect of Al on cellular ATPase activities (F_1F_0-ATPase, V-ATPase, P-ATPase) in wild type yeast. Values are means +/- SE (n=3). Results shown are representative of three independent trials.

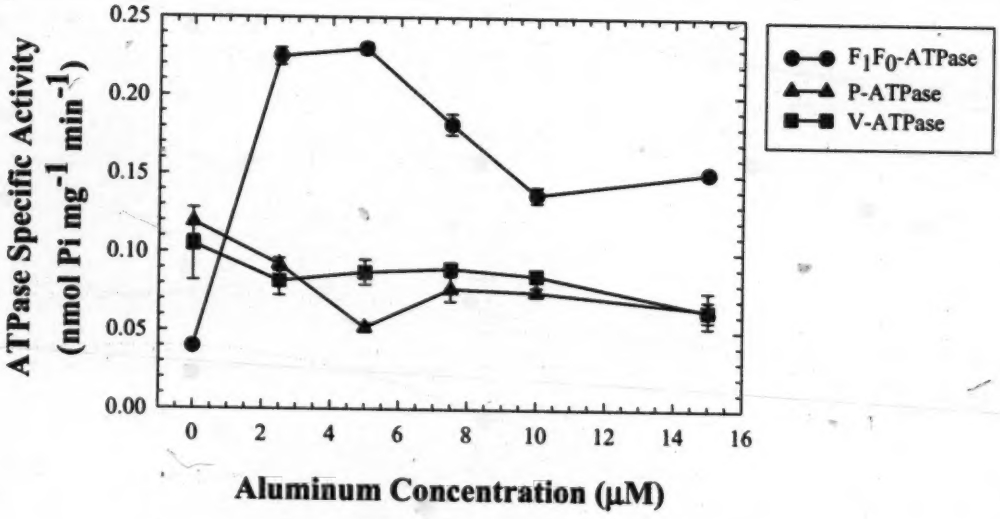
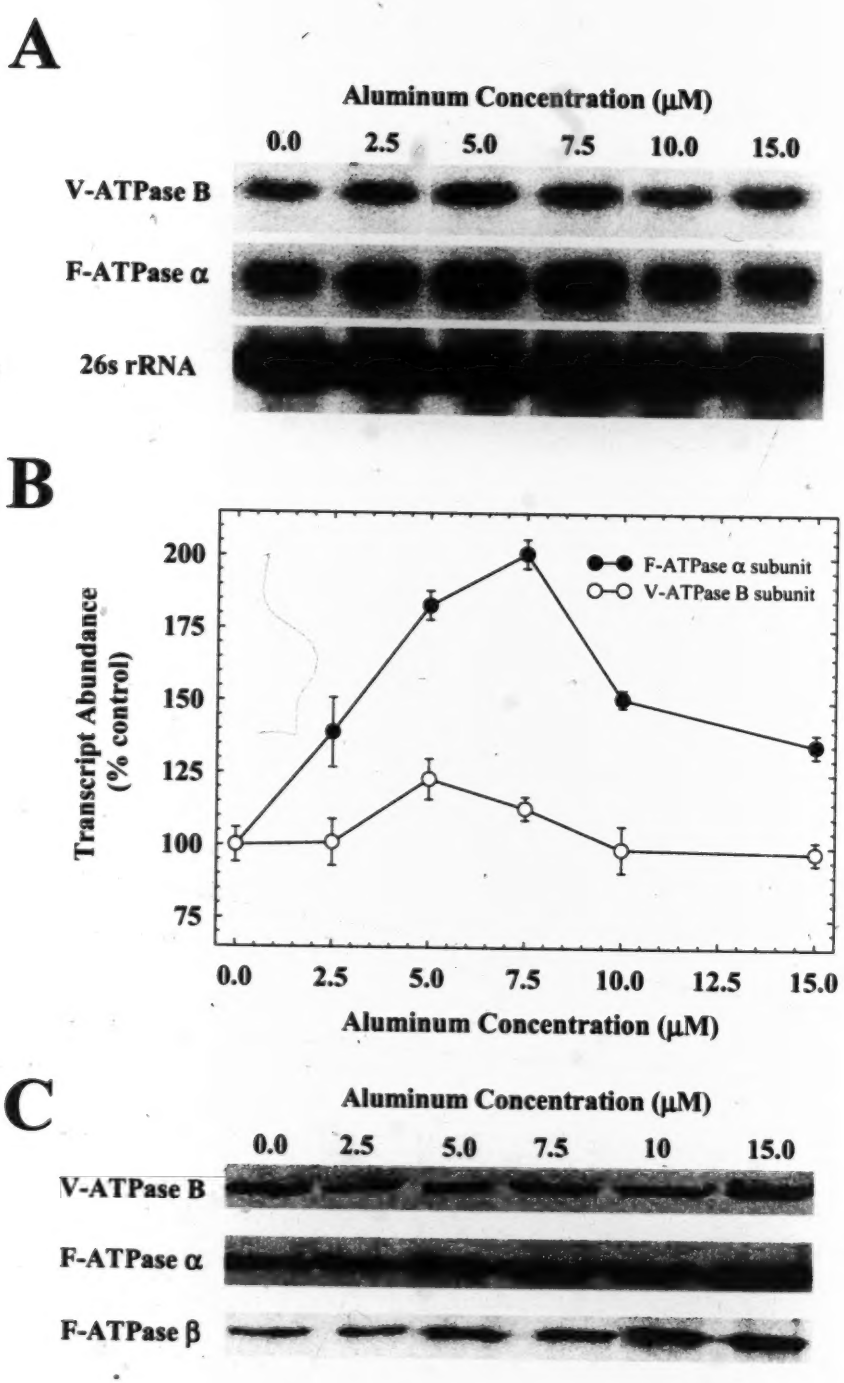

ATPase Specific Activity
(nmol Pi mg-1 min-1)
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
16
Aluminum Concentration (μM)
F1F0-ATPase
P-ATPase
V-ATPase

Fig 3-3. The effect of Al on expression of V-ATPase and F_1F_0-ATPase. (A) Levels of the transcripts encoding the F_1F_0-ATPase α subunit and V-ATPase B subunit were measured by Northern analysis. (B) Relative abundance of transcripts as measured by densitometry. The ratio of mRNA/rRNA was measured at each concentration of $AlCl_3$ and compared to the control values for each transcript. (C) Determination of protein levels of V-ATPase (B subunit) and F_1F_0-ATPase (α and β subunits) by Western analysis. Cross-reacting bands were visualized using a chemiluminescent detection system. Samples shown are representative lanes selected from triplicates compiled using Adobe Photoshop^R v 5.5. Values shown are means +/- SE (n=3) and are representative of 2 independent trials.


A
Aluminum Concentration (μM)
0.0
2.5
5.0
7.5
10.0
15.0
V-ATPase B
F-ATPase α
26s rRNA
B
Transcript Abundance (% control)
200
175
150
125
100
75
F-ATPase α subunit
V-ATPase B subunit
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Aluminum Concentration (μM)
C
Aluminum Concentration (μM)
0.0
2.5
5.0
7.5
10
15.0
V-ATPase B
F-ATPase α
F-ATPase β

subunits also increased with increasing $AlCl_3$ concentration (Fig 3-3C). Interestingly, levels of these subunits continue to increase past 5-7.5 μM $AlCl_3$, the peak concentrations for F_1F_0-ATPase activity and transcript levels.

Levels of the transcript encoding the V-ATPase B subunit remained nearly constant over the range of Al concentrations tested (Fig 3-3A,B), with only a slight increase to 123% of control at 5 μM $AlCl_3$. The amount of protein also remained constant (Fig 3-3C), reflecting the constant level of V-ATPase activity observed during Al stress (Fig 3-2). In wheat, V-ATPase transcript, protein and activity increased in parallel with increasing concentrations of Al (Hamilton *et al.*, 2001, C.A. Hamilton, unpublished data).

3.3.2 V-ATPase mutants, but not F_1F_0-ATPase mutants, are hypersensitive to Al

To test the hypothesis that V-ATPase and F_1F_0-ATPase activities are involved in Al resistance, yeast mutants lacking subunits of these enzymes were tested to determine whether or not they are hypersensitive to Al. The relative sensitivities of V-ATPase mutants, their parent strain and complemented strains to Al were determined by observing their growth in liquid culture. Cultures were grown in SC medium (pH 4.5) since *vma1* and *vma2* mutant strains grew poorly in the low phosphate, low pH medium, LPP (Schott and Gardner, 1997), normally used for experiments involving Al. In the V-ATPase mutant strains (*vma1, vma2*), there was a striking 40% decrease in growth (from 0.85 and 1.09 control OD_{600} to 0.45 and 0.62 OD_{600} at 75 μM $AlCl_3$, respectively) at all Al concentrations tested, while the parent

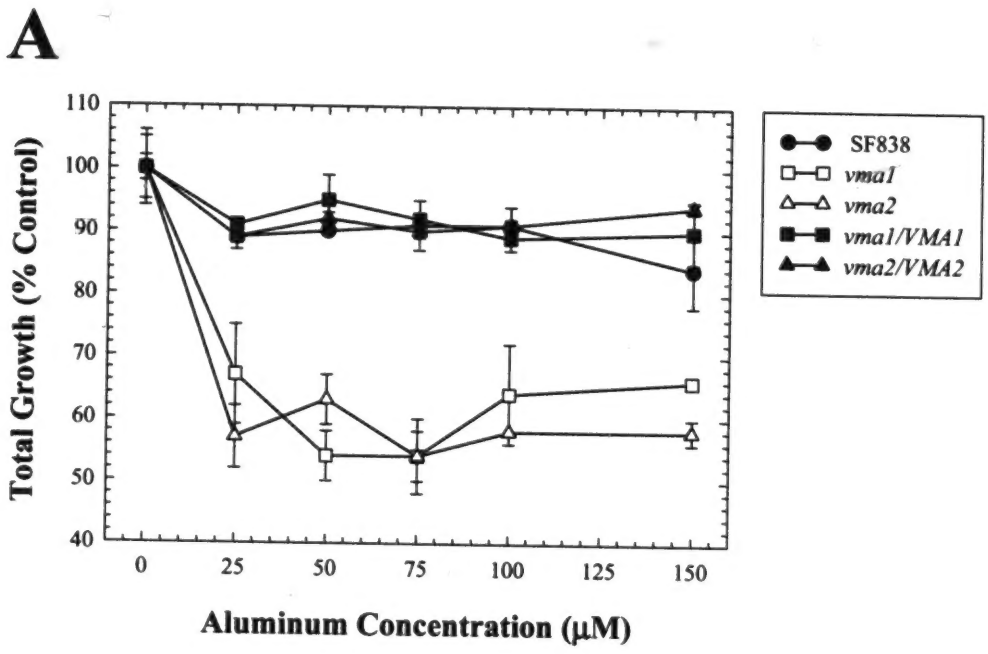
strain (control OD_{600} 1.28) was unaffected (Fig 3-4a). The complemented strains (control OD_{600} values of 1.15 and 1.19, respectively) were similarly unaffected by Al. This confirmed that the V-ATPase mutations are responsible for the Al sensitive phenotype of the *vma1* and *vma2* strains.

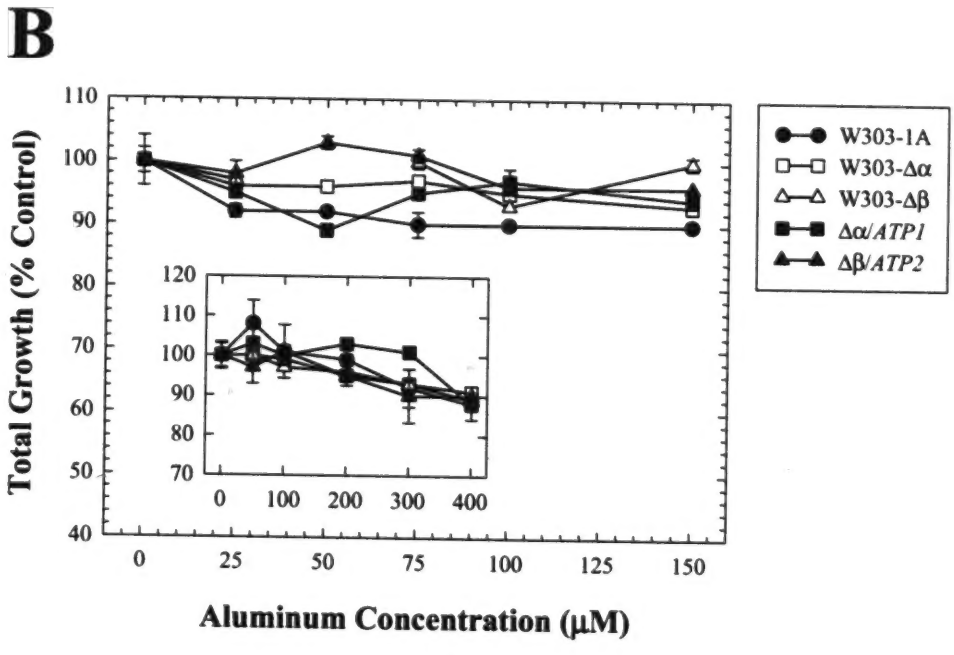
The relative sensitivities of F_1F_0-ATPase mutants, their parent strain and complemented strains to Al were also measured (Fig 3-4B). In contrast to V-ATPase mutants, F_1F_0-ATPase mutants did not show hypersensitivity to Al from 0-150 μM. To determine whether higher concentrations were required to observe phenotypic differences, levels of Al up to 400 μM were tested (Fig 3-4B, inset). No discernable differences were observed between the five strains.

3.4 Discussion

The responses of plants and yeast to Al are somewhat different. For example, Al toxicity in yeast can be overcome by overexpression of a Mg^{2+} transporter (MacDiarmid and Gardner, 1998), presumably due to inhibition of Mg^{2+} transport by Al^{3+}. In contrast, although addition of Mg^{2+} (or other divalent cations such as Ca^{2+}) alleviates Al^{3+} toxicity in wheat (Kinraide, 1998), reduced Mg^{2+} availability does not cause Al toxicity symptoms (Kinraide and Parker, 1987). Also, many gene products induced by Al in plants have no protective effect against Al when expressed in yeast (Ezaki *et al.*, 1999). This is likely because many Al-induced proteins are general stress response proteins (induced in response to oxidative stress and heat shock) which do not necessarily have a role in Al resistance. Therefore, physiological and

Fig 3-4. The effect of ATPase mutations on Al resistance in yeast. (a) Yeast strains carrying LEU2 insertion mutations in the *VMA1* and *VMA2* genes (*vma1*, *vma2*), their isogenic parent strain (SF838), and strains carrying wild type copies of the *VMA1* and *VMA2* genes on low copy plasmids (*vma1/VMA1*, *vma2/VMA2*) were grown for 16-24 h in SC minimal medium (pH 4.5) containing 0-150 μM $AlCl_3$. (b) Yeast strains with null deletion mutations in the *atp1* and *atp2* genes (W303-Δα, W303-Δβ), their isogenic wild type parent strain (W303-1A) and strains carrying wild type copies of the *atp1* and *atp2* genes on low copy plasmids (Δα-*ATP1*, Δβ-*ATP2*) were grown for 16-24 h in SC minimal medium (pH 4.5) containing 0-150 μM $AlCl_3$ (the inset shows that similar results were observed up to 400 μM $AlCl_3$). Total growth (OD_{600}) of each culture was measured and compared to the control (0 $AlCl_3$) values for each strain. Values shown are means +/- SE (n=3). Results shown are representative of three independent trials.


A
Total Growth (% Control)
Aluminum Concentration (μM)
SF838
vma1
vma2
vma1/VMA1
vma2/VMA2


B
Total Growth (% Control)
Aluminum Concentration (μM)
W303-1A
W303-Δα
W303-Δβ
Δα/ATP1
Δβ/ATP2

structural differences between yeast and plants must be considered when analyzing data from this system.

It was recently shown by Ezaki *et al.* (1998) that yeast cells exposed to Al show irregular vacuolar morphology. The authors did not speculate as to the possible cause of this effect, but it is possible that Al associated with the yeast vacuole causes this phenotype. Aluminum uptake has been demonstrated in yeast (Jagannatha *et al.*, 1997; Ezaki *et al.*, 1999) and the giant algae *Chara corallina* (Taylor *et al.*, 2000), where it is associated mainly with the cell wall, but is also taken up into the vacuole. Ezaki *et al.* (1999) suggested that intracellular Al does not associate with the yeast vacuole based on observations of morin fluorescence, but low levels such as those measured in *Chara* (<5 $\mu g\ m^{-2}$, Taylor *et al.*, 2000) would not be detected using this method. It is possible that Al is sequestered in the vacuole by an active transport mechanism which relies on the tonoplast electrochemical potential. Once in the vacuole, Al could be effectively sequestered by complexing with polyphosphate (as is calcium) (Dunn *et al.*, 1994) or organic anions.

Here I have used the yeast model system for simple and direct testing of a hypothesis derived from higher plants. Based on experiments in plants, I hypothesized that the V-ATPase is involved in Al resistance, with the ATP required for its activity supplied by increased activity of ATP synthase. Although V-ATPase transcript and protein levels, as well as enzyme activity, are not affected by increasing Al concentrations in yeast as they are in plants, the observation that V-ATPase mutants are hypersensitive to Al supports my hypothesis. In wild-type yeast, F_1F_0-

ATPase transcript, protein and activity levels all increase in response to Al, while F_1F_0-ATPase mutants do not show an Al hypersensitive phenotype. It is possible that the demand for ATP can be met by fermentation, although ATP synthase would normally fulfill this requirement in wild-type yeast and plants.

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4. Vacuolar H^+-ATPase, but not Mitochondrial F_1F_0-ATPase, is Required for NaCl Tolerance in *Saccharomyces cerevisae*[3]

4.1 Introduction

Salt tolerance is a complex trait involving responses to both osmotic and ionic stresses. The mechanisms by which organisms are able to tolerate high concentrations of salt in their environment have been extensively studied by mutational analysis of the budding yeast *Saccharomyces cerevisiae*. Data obtained thus far indicate that an array of signaling pathways activate salt-responsive genes involved in modulating plasma membrane polarization, Na^+ influx, Na^+ efflux and sequestration of Na^+ in the vacuole.

One effect of salt stress is a hyperpolarization of the plasma membrane. A low affinity cation uptake current activated by membrane hyperpolarization (NSC1) has been observed in patch clamp studies (Bihler *et al.*, 1998) and may contribute to subsequent depolarization of the membrane. Also contributing to depolarization of the plasma membrane are protein kinases HAL4 and HAL5, the induction of which increases the selectivity of the high affinity TRK1/2 K^+ channel (Mulet *et al.*, 1999). Influx of K^+ and H^+ (and perhaps Na^+) through this channel dissipates the driving force for uptake of Na^+ across the plasma membrane. The Ser-Thr protein

[3] A version of this chapter has been submitted for publication. Hamilton CA, Taylor GJ and Good AG (2001) Vacuolar H^+-ATPase, but not Mitochondrial F_1F_0-ATPase, is Required for NaCl Tolerance in *Saccharomyces cerevisae*. FEMS Microbiol Lett (In Press)

phosphatase, calcineurin, is also required for increased selectivity of TRK1/2 (Mendoza *et al.*, 1994). Calcineurin is a heterotrimer composed of a catalytic subunit (encoded by either *CNA1*or *CNA2*), a Ca^{2+}-binding regulatory subunit (encoded by *CNB1*) and Ca^{2+}-calmodulin (Stemmer and Klee, 1994). Binding of Ca^{2+}/calmodulin to the catalytic subunit prevents its autoinhibitory activity (Cohen, 1989) so that calcineurin is free to interact with a variety of substrates within the cell, regulating the salt response.

Calcineurin is a central regulator of Na^+ influx and efflux. Sodium ions may enter the cell through the TRK1/2 channel or the NSC1 current. Under alkaline or neutral conditions, Na^+ is exported mainly through the action of PMR2/ENA1, a Na^+-ATPase localized to the plasma membrane (Prior *et al.*, 1996). Expression and activity of this enzyme is controlled by many factors, highlighting its importance to salt tolerance. Activity of ENA1 is stimulated by calcineurin (Cunningham and Fink, 1996), HAL1,8,9 (Gaxiola *et al.*, 1992; Rios *et al.*, 1997; Mendizabal *et al.*, 1998), SNF1 (Rios *et al.*, 1997), and in *Schizosaccharomyces pombe*, AFT1, a transcription factor activated by the *STY1/WIS1* MAPK cascade (Nishikawa *et al.*, 1999). It is also indirectly activated by HAL3, a negative regulatory subunit of PPZ1/2 protein phosphatases, which normally inhibit ENA1 activity (Posas *et al.*, 1995). Carbon source also affects ENA1 activity. The presence of glucose inhibits SNF1 (an activator of ENA1) and activates protein kinase A, an inhibitor of ENA1 (Rios *et al.*, 1997). Under acidic conditions such as those used in this study, efflux of Na^+ is mediated mainly by the Na^+/H^+ antiporter, NHA1(Prior *et al.*, 1996).

Another mechanism of salt tolerance in yeast is sequestration in the vacuole. Transport of Na^+ into the vacuole is mediated by NHX1, a Na^+/H^+ antiporter localized to a prevacuolar compartment and activated by cytoplasmic acidosis (Nass *et al.*, 1997; Nass and Rao, 1998). Other proteins required for *NHX1*-mediated salt tolerance are the chloride channel (GEF1) and the vacuolar ATPase (Gaxiola *et al.*, 1999).

Since transmembrane transport of Na^+ is a key component of salt tolerance, the objective of the present study was to characterize the responses of cellular ATPases (P-ATPase, F_1F_0-ATPase and V-ATPase) to salt. The contributions of the V-ATPase and F_1F_0-ATPase were of particular interest because the V-ATPase (with ATP provided by F_1F_0-ATPase) creates the proton motive force (PMF) required for import of cations into the vacuole. Changes in ATPase transcript levels, protein and activity in response to salt were measured, and the phenotypes of V-ATPase and F_1F_0-ATPase mutant and complemented strains were compared.

4.2 Materials and Methods

4.2.1 Media and culture conditions

Yeast strains (Table 3-1) were maintained on standard media as described by Sherman, 1991.

4.2.2 ATPase activity measurements

Seed cultures (3 ml in synthetic complete medium, SC) of wild-type yeast were inoculated from YPD plate stocks and incubated for 16-24 h at 30°C. Cells were

harvested by centrifugation, washed three times and resuspended in 1 ml of sterile water. This cell suspension was used to inoculate triplicate 3 ml cultures of SC medium containing 0-1000 mM NaCl to a starting OD_{600} of approximately 0.01 and cultures were incubated for 16-24 h at 30°C (final OD_{600} approximately 0.9). Each 3 ml culture was harvested by centrifugation and spheroplasts were prepared as described by Uchida *et al.*, 1988. Cell suspensions were then sonicated at low intensity (Branson Sonifier, 10% output) for 30 s to gently lyse cells. Cell debris was removed by centrifugation and the resulting supernatant used for enzyme assays. Quantitation of protein in cell extracts was performed using the Bradford assay (Bradford, 1976). Adenosine triphosphatase activity was then assayed as described by Briskin *et al.*, 1987.

4.2.3 Complementation of ATPase mutations

Yeast strains carrying *LEU2* insertion mutations in *vma1* and *vma2*, as well as plasmids carrying the complementing genes, were provided by Dr Tom Stevens (University of Oregon). Strains carrying null deletion mutations of *atp1* and *atp2* and plasmids carrying the complementing genes were provided by Dr David Mueller (Chicago Medical School). In order to select for transformation of the plasmids in the appropriate mutant strains, *ATP1* and *VMA2* were recloned into pRS314 (TRP1) and pRS316 (URA3) and transformed into appropriate yeast strains as described by Hamilton *et al.*, 2001 (Chapter 2).

4.2.4 NaCl dose response experiments

Inoculum for each culture was prepared as described above and used to inoculate triplicate 3 ml cultures of SC medium containing 0-1000 mM NaCl to a starting OD_{600} of approximately 0.01. Cultures were incubated for 16-24 h at 30°C and the final OD_{600} of each culture was measured in a 96-well plate (Costar) using a Spectromax Plus plate reader and Softmax Pro v 1.1 software (Molecular Devices). For comparison, the OD_{600} values for each strain were expressed as a percentage of the control value for that strain. For halotolerance tests, 5 µL aliquots of serial 10^{-1} dilutions of overnight cultures were spotted onto YPD medium containing 1 M NaCl, 1.5 M sorbitol, 100 mM LiCl or 50 µg ml^{-1} hygromycin B and incubated at 30°C for 2-4 d.

4.2.5 Immunoblotting

Total yeast proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose (0.45 µm, BioRad Laboratories) membranes using the Mini Trans-Blot Cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked and probed as directed in the Lumi-glo Chemiluminescent Detection System manual (Kirkegaard and Perry Laboratories). Polyclonal antibodies raised against the α and β subunits of the yeast F_1-ATPase were provided by Dr A. Lewin, University of Florida. Antibodies raised against the B subunit of the *Vigna radiata* V-ATPase were provided by Dr M. Maeshima, Nagoya University.

4.2.6 RNA Isolation, Northern Hybridization and Analysis

RNA was isolated from yeast cultures (approximately $5x10^7$ cells) using the RNeasy Kit (Qiagen) according to the manufacturer's directions. Northern transfers were carried out using GeneScreen Plus (DuPont) membranes according to the manufacturer's directions. Probes for Northern blots were isolated from pRS316VMA2 and pRS316ATP1 by digestion with appropriate restriction enzymes (Amersham Pharmacia Biotech) and inserts were isolated from agarose gels using the QiaQuick gel extraction kit (Qiagen). Probes were then prepared by random priming. Membranes were prehybridized, hybridized and washed according to the manufacturer's directions, followed by exposure to storage phosphor screens (Molecular Dynamics). Phosphor screens were developed using a Phosphorimager 445SI and the resulting images analyzed using ImagequaNT v 4.2 software. For figure preparation, representative lanes of triplicate samples were selected from the same blot and compiled using Adobe Photoshop^R version 5.5.

4.3 Results

4.3.1 Response of yeast ATPases to NaCl

The responses of cellular ATPases (F_1F_0-ATPase, P-ATPase and V-ATPase) to NaCl in wild type yeast were characterized by measuring ATPase activities, protein and transcript levels. Adenosine triphosphatase activities were measured after growth

in SC medium containing 0-1000 mM NaCl (Fig 4-1). F_1F_0-ATPase activity gradually increased from 0.078 nmol Pi mg^{-1} min^{-1} to 0.154 nmol Pi mg^{-1} min^{-1} (197% of control), reaching its peak activity at 600 mM NaCl. P-ATPase activity remained essentially constant from 0-800 mM NaCl, but declined to 0.056 nmol Pi mg^{-1} min^{-1} (57% of control) by 1000 mM. At first sight this is surprising, since the main determinant of NaCl tolerance in yeast is ordinarily the ENA1 Na^+ efflux pump (a P-type ATPase). However, under the acidic conditions used here (pH 4.5), Na^+/H^+ antiport mediated by NHA1 would predominate (Prior *et al.*, 1996). The presence of glucose in the growth medium would also inhibit the activity of ENA1 by repression of SNF1, an activator of ENA1, and by activation of protein kinase A, an inhibitor of ENA1 (Rios *et al.*, 1997).

In contrast, vacuolar ATPase activity increased sharply from 0.103 nmol Pi mg^{-1} min^{-1} to 0.303 nmol Pi mg^{-1} min^{-1} (296% of control) from 0-200 mM NaCl, and decreased slightly at higher salt concentrations. However, even at 1000 mM NaCl, V-ATPase activity was still comparatively high, at 0.194 nmol Pi mg^{-1} min^{-1} (189% of control).

To determine the mechanism by which ATPase activities change during NaCl stress, expression of V-ATPase and F_1F_0-ATPase subunits was examined by Northern and Western analysis (Fig 4-2). Levels of the transcript encoding the F_1F_0-ATPase α subunit increased to 325% of the control level by 800 mM NaCl, followed by a decline to 257% of control by 1000 mM (Fig 4-2A,B). This pattern is similar to that observed for increased F_1F_0-ATPase activity (Fig 4-1), with the exception that

Fig 4-1. The effect of NaCl on cellular ATPase activities (F_1F_0-ATPase, P-ATPase, V-ATPase) in wild type yeast. Cultures were grown 16-24 h in the presence of 0-1000 mM NaCl in SC medium. Values shown are means +/- SE (n=3). Results shown are representative of three independent trials.

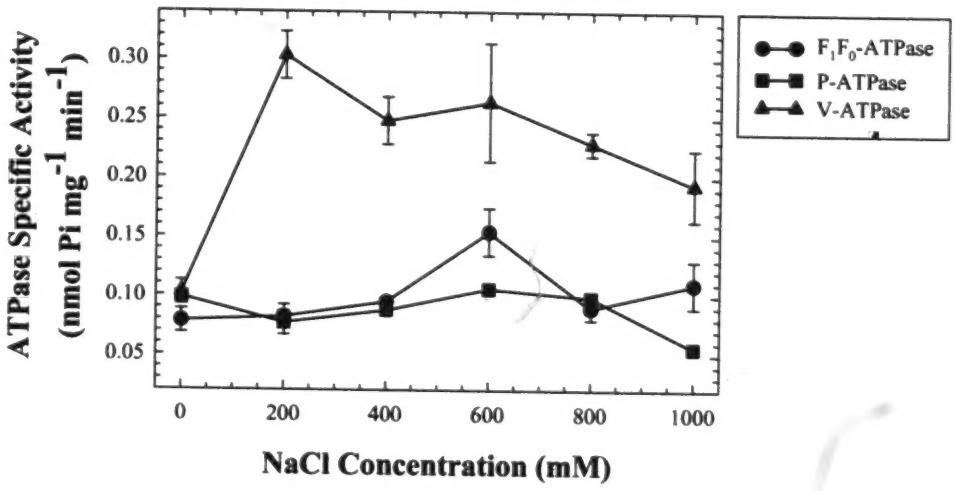
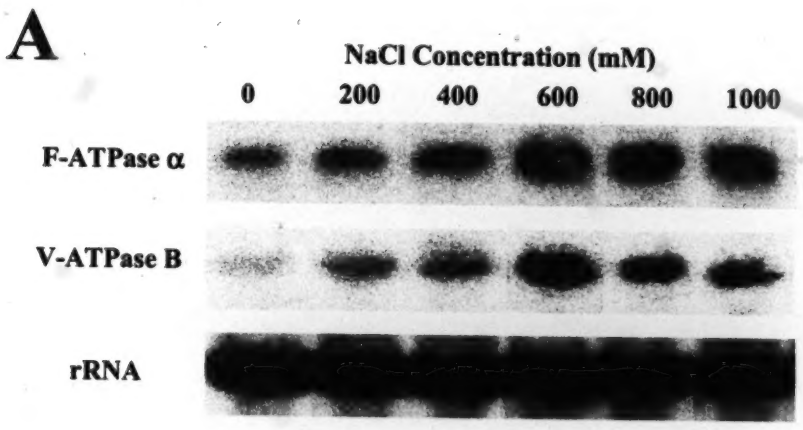
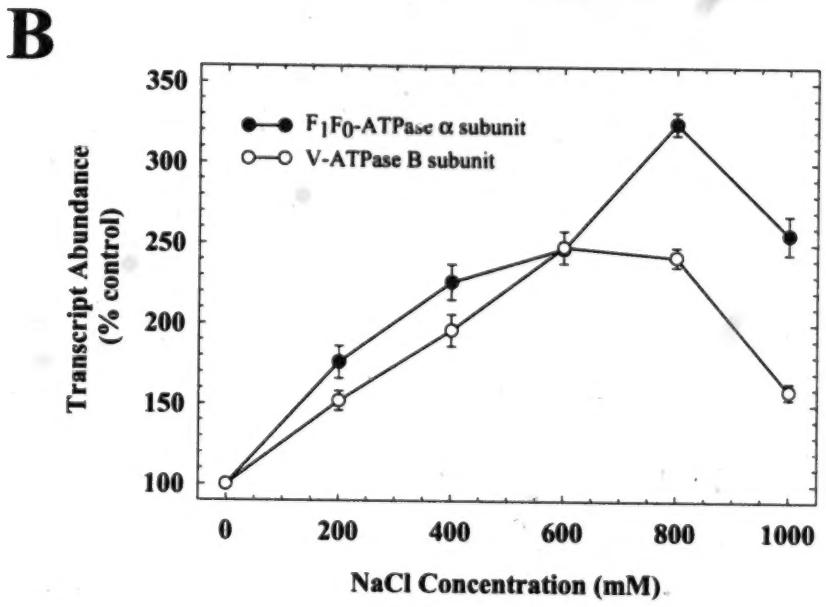
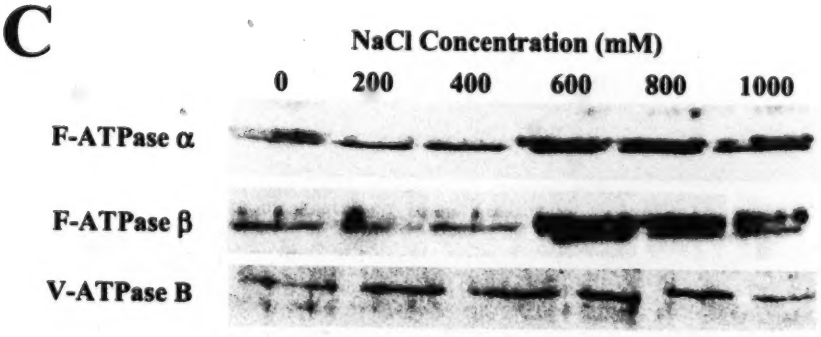

ATPase Specific Activity
(nmol Pi mg-1 min-1)
0.30
0.25
0.20
0.15
0.10
0.05
0
200
400
600
800
1000
NaCl Concentration (mM)
F1F0-ATPase
P-ATPase
V-ATPase

Fig 4-2. The effect of NaCl on expression of F_1F_0-ATPase and V-ATPase. (A) Levels of the transcripts encoding the F_1F_0-ATPase α subunit and V-ATPase B subunit were measured by Northern analysis. (B) Relative abundance of transcripts as measured by densitometry. The ratio of mRNA/rRNA measured at each concentration of NaCl was compared to the control value for each transcript. Values shown are means +/- SE (n=3). (C) Determination of protein levels of V-ATPase (B subunit) and F_1F_0-ATPase (α and β subunits) by Western analysis. Cross-reacting bands were visualized using a chemiluminescent detection system. Samples shown are representative lanes selected from triplicates and compiled using Adobe Photoshop^R v 5.5. For all panels, data shown are representative of 2 independent trials.


A
NaCl Concentration (mM)
0
200
400
600
800
1000
F-ATPase α
V-ATPase B
rRNA


B
350
300
250
200
150
100
F1F0-ATPase α subunit
V-ATPase B subunit
Transcript Abundance
(% control)
0
200
400
600
800
1000
NaCl Concentration (mM)


C
NaCl Concentration (mM)
0
200
400
600
800
1000
F-ATPase α
F-ATPase β
V-ATPase B

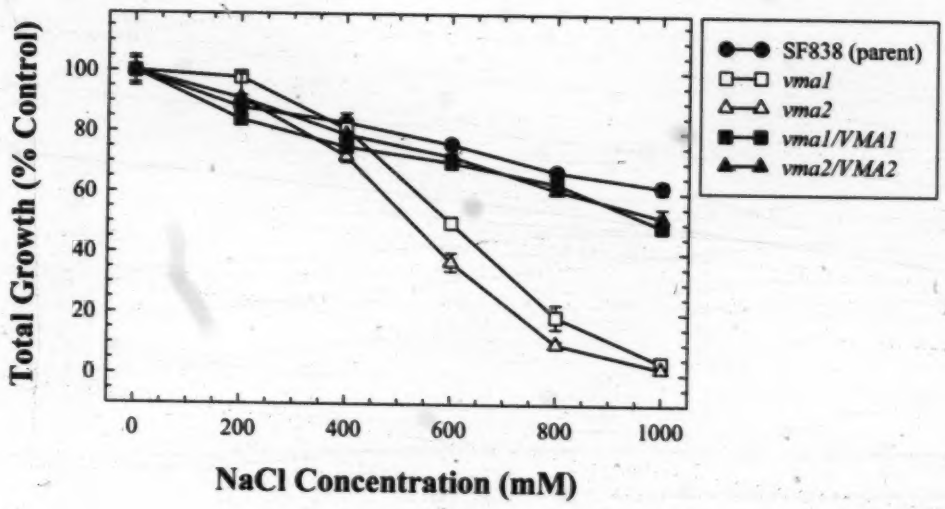
enzyme activity peaks at 600 mM while the transcript level peaks at 800 mM. Protein levels of the F_1F_0-ATPase α and β subunits also increased with increasing NaCl concentration, as determined by Western analysis (Fig 4-2C). Interestingly, protein levels of these subunits peak at 600-800 mM NaCl, which are also the peak concentrations for F_1F_0-ATPase transcript levels. Levels of the transcript encoding the V-ATPase B subunit also increased gradually over the range of NaCl concentrations tested (Fig 4-2A,B), reaching a peak (248% of control) at 600 mM NaCl. The amount of protein also increased in response to NaCl stress. Like the α and β subunits of the F_1F_0-ATPase, protein levels of the V-ATPase B subunit continued to increase from 0-800 mM NaCl (Fig 4-2C), in contrast to the peak levels of enzyme activity and transcript abundance (200 mM and 600 mM NaCl, respectively).

4.3.2 V-ATPase mutants, but not F_1F_0-ATPase mutants, are hypersensitive to NaCl

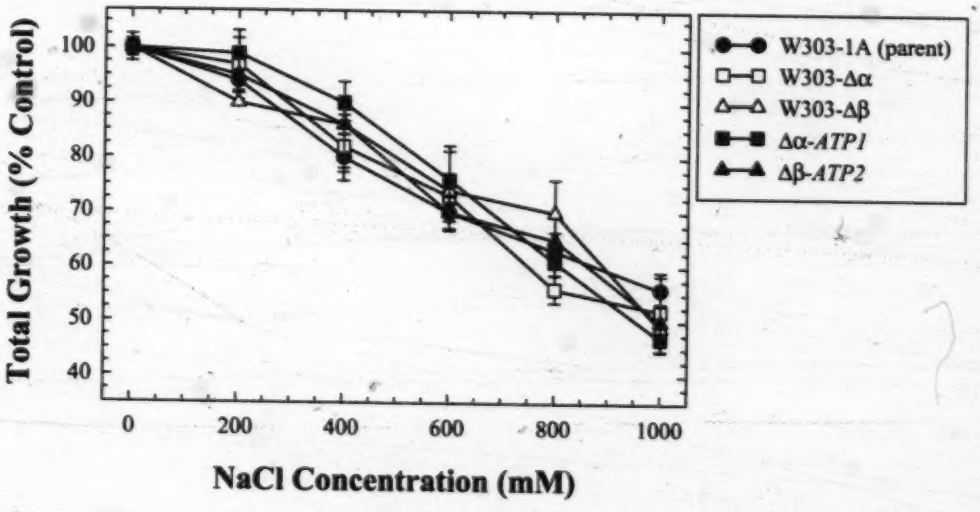
To test the hypothesis that V-ATPase and F_1F_0-ATPase activities are involved in salt tolerance, yeast mutants lacking subunits of these enzymes were tested to determine whether or not they are hypersensitive to NaCl. The relative sensitivities of V-ATPase mutants, wild-type parent and complemented strains to NaCl were determined by observing growth in liquid culture. Final OD_{600} values at each NaCl concentration were related to the control values for each strain (Fig 4-3A). For each V-ATPase mutant strain (*vma1, vma2*), there was a 40-50% greater decrease in growth compared to wild-type at 600-1000 mM NaCl (from control OD_{600} values of

Fig 4-3. The effect of ATPase mutations on NaCl tolerance in yeast. (A) Yeast strains carrying LEU2 insertion mutations in the *VMA1* and *VMA2* genes (*vma1*, *vma2*), their isogenic parent strain (SF838), and strains carrying wild type copies of the *VMA1* and *VMA2* genes on low copy plasmids (*vma1/VMA1*, *vma2/VMA2*) were grown for 16-24 h in SC minimal medium (pH 4.5) containing 0-1000 mM NaCl. (B) Yeast strains with null deletion mutations in the *atp1* and *atp2* genes (W303-Δα, W303-Δβ), their isogenic wild type parent strain (W303-1A) and strains carrying wild type copies of the *atp1* and *atp2* genes on low copy plasmids (Δα-*ATP1*, Δβ-*ATP2*) were grown for 16-24 h in SC minimal medium (pH 4.5) containing 0-1000 mM NaCl. Total growth (OD_{600}) of each culture was measured and compared to the control (0 NaCl) values for each strain. Values shown are means +/- SE (n=3). Results shown are representative of three independent trials.

A


Total Growth (% Control)
100
80
60
40
20
0
0
200
400
600
800
1000
NaCl Concentration (mM)
SF838 (parent)
vma1
vma2
vma1/VMA1
vma2/VMA2

B

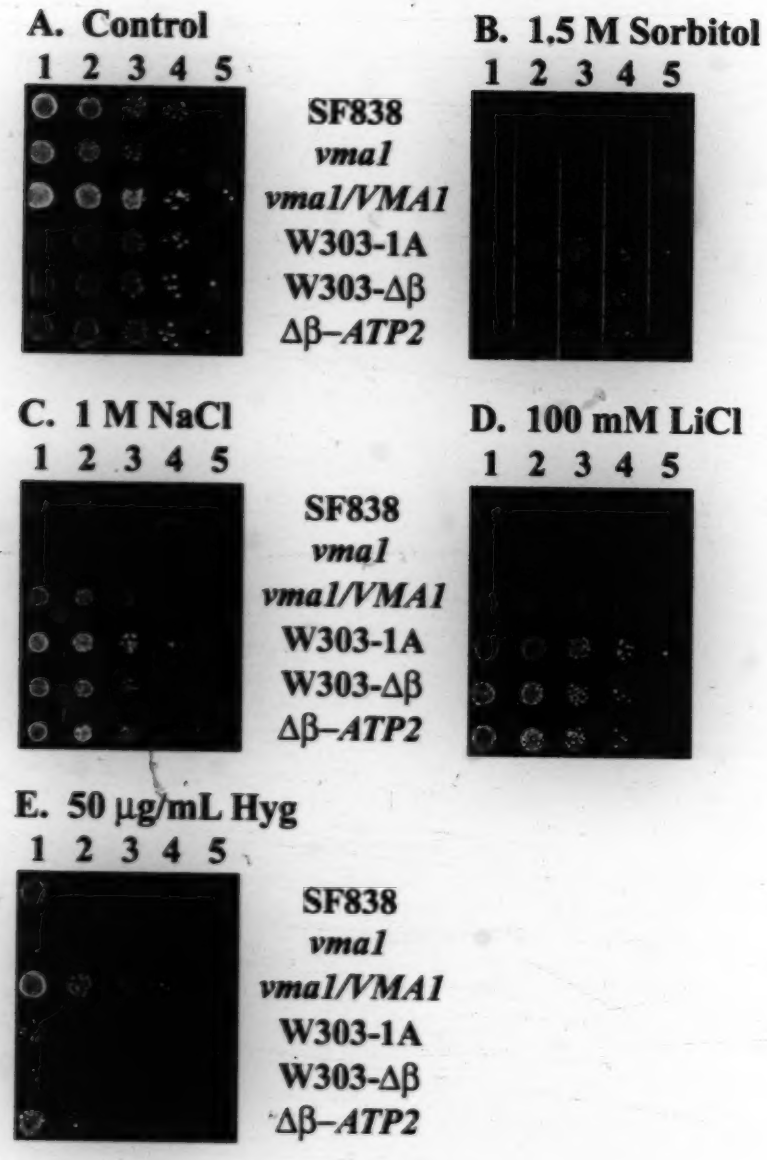

Total Growth (% Control)
100
90
80
70
60
50
40
0
200
400
600
800
1000
NaCl Concentration (mM)
W303-1A (parent)
W303-Δα
W303-Δβ
Δα-ATP1
Δβ-ATP2

0.49 and 0.48 to OD_{600} values of 0.08 and 0.05, for *vma1* and *vma2* respectively at 800 mM NaCl). In contrast, the complemented strains grew nearly as well as wild-type at these concentrations (control OD_{600} values of 0.82, 0.73 and 0.76 and OD_{600} values of 0.55, 0.46 and 0.46 at 800 mM NaCl for SF838, *vat1/VAT1* and *vat2/VAT2* respectively).

The salt-sensitive phenotype observed for the V-ATPase mutant strain, *vma1*, was specific to NaCl and LiCl (Fig 4-4). This mutant grew as well as the wild-type parent and complemented strains on YPD medium containing other osmotic (sorbitol) and ionic (hygromycin B) stress-inducing agents. In contrast, hypersensitivity of V-ATPase mutants was observed on YPD medium containing 1 M NaCl or 100 mM LiCl (Fig 4-4).

The relative sensitivities of F_1F_0-ATPase mutant strains, parent and complemented strains (W303-Δα, W303-Δβ, W303-1A, Δα-*ATP1* and Δβ-*ATP2*) to NaCl were also measured (Fig 4-3B). In contrast to the V-ATPase mutants, the F_1F_0-ATPase mutants were not hypersensitive to NaCl from 0-1000 mM (control OD_{600} values of 0.87, 0.79, 0.73, 0.83, and 0.87 for W303-1A, W303-Δα, W303-Δβ, Δα-*ATP1* and Δβ-*ATP2*, respectively). Growth of the W303-Δβ mutant strain was indistinguishable from its parent and complemented strains in the presence of hygromycin B, LiCl, sorbitol and NaCl on YPD solid medium (Fig 4-4).

Fig 4-4. Halotolerance of V-ATPase and F_1F_0-ATPase mutant strains. Saturated cultures of wild-type parent (SF838, W303-1A), mutant (*vma1*, W303-Δβ) and ccomplemented (*vma1/VMA1*, Δβ-*ATP2*) strains were serially diluted and 5 μL aliquots of 10^{-1} to 10^{-5} dilutions were spotted onto YPD plates containing (A) no added compounds, (B) 1.5 M sorbitol, (C) 1 M NaCl, (D) 100 mM LiCl or (E) 50 μg ml^{-1} hygromycin B. Results shown are representative of two independent trials.


A. Control
1 2 3 4 5
B. 1.5 M Sorbitol
1 2 3 4 5
SF838
vma1
vma1/VMA1
W303-1A
W303-Δβ
Δβ–ATP2
C. 1 M NaCl
1 2 3 4 5
D. 100 mM LiCl
1 2 3 4 5
SF838
vma1
vma1/VMA1
W303-1A
W303-Δβ
Δβ–ATP2
E. 50 μg/mL Hyg
1 2 3 4 5
SF838
vma1
vma1/VMA1
W303-1A
W303-Δβ
Δβ–ATP2

4.4 Discussion

This chapter highlights the importance of the vacuolar ATPase in yeast NaCl tolerance. In wild-type yeast, NaCl stress causes increased activity of the V-ATPase, with ATP presumably provided by increased activity of the F_1F_0-ATPase. The V-ATPase is thought to contribute to NaCl tolerance by creating the PMF required for sequestration of Na^+ in the vacuole by the Na^+/H^+ antiporter, NHX1 (Nass *et al.*, 1997). The electrical potential created by the V-ATPase also creates a driving force for uptake of Cl^- through the chloride channel (GEF1). Therefore, it is possible that V-ATPase mutant strains are hypersensitive to NaCl because they are less able to effectively sequester Na^+ and Cl^- in the vacuole, allowing Na^+ to interact with sensitive cytoplasmic targets. This hypothesis is supported by recent experiments in which the vacuolar H^+-pyrophosphatase (AVP1) from *Arabidopsis thaliana* was overexpressed in yeast and in *Arabidopsis*. Overexpression of this enzyme mimics overexpression of the V-ATPase, and in both species resulted in enhanced NaCl tolerance (Gaxiola *et al.*, 1999, 2001). Further support for the involvement of the vacuole in NaCl tolerance comes from the observation that overexpression of the vacuolar Na^+/H^+ antiporter also confers enhanced NaCl tolerance in yeast (Gaxiola *et al.*, 1999) and *Arabidopsis* (Apse *et al.*, 1999).

Interestingly, my observation that V-ATPase activity is stimulated by NaCl in wild-type yeast contrasts observations by Perzov *et al.* (2001). These authors examined vacuoles isolated from yeast cells (strain W303-1A) grown in YPD medium containing 800 mM NaCl and found that proton uptake activity was

decreased in comparison to activity in vacuoles isolated from cells grown in medium without NaCl. However, the differences in medium composition (YPD vs SC) and pH (neutral vs acidic) make direct comparison difficult, possibly highlighting the importance of environment in determining which salt tolerance mechanisms are active.

My observation that V-ATPase mutants were inhibited 40-50% more than the wild-type parent and complemented strains at 600-1000 mM NaCl is consistent with those obtained by Gaxiola *et al.* (1999), who found that overexpression of the *Arabidopsis* vacuolar H^+-pyrophosphatase led to increased NaCl tolerance. Haro *et al.* (1993) examined the NaCl tolerance of yeast strains lacking the C subunit of the V-ATPase. Although my observation that V-ATPase mutants are hypersensitive to NaCl is in agreement with their results, they also reported that these mutants tolerate only 15 mM LiCl, contrary to our observations. I observed hypersensitivity at 100 mM LiCl but not at 50 mM LiCl (data not shown). It is possible that these differences arose due to the different media used (YPD vs minimal medium arginine phosphate) or because I performed growth tests on solid medium while Haro *et al.* used liquid medium. In my experience, V-ATPase mutant strains grow poorly in liquid culture because the cells aggregate and have a slow growth rate. These confounding effects, caused by the pleiotropic nature of V-ATPase mutations, are not apparent when these strains are grown on solid medium.

Although it was previously known from genetic studies that V-ATPase mutants are hypersensitive to NaCl stress (Haro *et al.*, 1993), a detailed study of the

effect of NaCl on ATPase activity and expression in wild-type yeast has not been described in the literature. This chapter showed that NaCl induces activities of V-ATPase and F_1F_0-ATPase, and represses activity of the P-ATPase at high NaCl concentrations (1 M). Transcript and protein levels of the α and β subunits of the F_1F_0-ATPase and the B subunit of the V-ATPase also increased with NaCl treatment. I have also shown that under the same conditions that normally cause induction of V-ATPase activity (up to 1 M NaCl in SC medium pH 4.5), V-ATPase mutant strains exhibit NaCl hypersensitivity compared to wild-type parent and complemented strains. This hypersensitivity is restricted to NaCl and LiCl, since no hypersensitivity is observed when the same strains are grown on sorbitol or hygromycin B. Since V-ATPase activity is required for NaCl tolerance, as well as Al tolerance as described in chapter 3, future testing of the energy balance hypothesis will involve measurement of Al and NaCl tolerance in transgenic plants.

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Yamashiro CT, Kane PM, Wolczyk DF, Preston RA and Stevens TH (1990) Role of vacuolar acidification in protein sorting and zymogen activation: A genetic analysis of the yeast vacuolar proton-translocating ATPase. Mol Cell Biol. **10**: 3737-3749

5. Involvement of the Vacuolar H^+-ATPase in Al and NaCl Tolerance in *Arabidopsis thaliana*

5.1 Introduction

Aluminum tolerance in *Arabidopsis thaliana* has been studied mainly from a genetic perspective. Mutagenesis has been used to generate Al-resistant and Al-sensitive mutants of *Arabidopsis*, some of which have been characterized. Larsen *et al.* (1997a) isolated 9 *als* (*al*uminum *s*ensitive) mutants corresponding to 8 unique loci and 7 *alr* (*al*uminum *r*esistant) mutants corresponding to 2 unique loci. One of the *als* mutants, *als*-3, caused inhibition of shoot development, leaf expansion and root growth in response to Al (Larsen *et al.*, 1997b). The genetic and physiological basis for this mutant phenotype has not been determined, and mutants representing the other 7 *als* loci have not yet been described in the literature. One of the *alr* loci, represented by the *alr*-108, *alr*-128 and *alr*-131 alleles, is associated with increased exudation of citrate, malate and pyruvate in response to Al, which may be caused by altered organic acid metabolism or transport (Larsen *et al.*, 1998). Increased exudation of organic anions is thought to contribute to detoxification of Al in the rhizosphere (Taylor, 1991). The other *alr* locus, represented by the *alr*-104 allele, is associated with alkalinization of the rhizosphere in response to Al. After Al

exposure, H^+ influx across the plasma membrane is increased 2-fold in the *alr*-104 mutant compared to wild-type. The Al resistant phenotype is not observed in buffered solutions, suggesting that alkalinization of the rhizosphere is responsible for the Al resistance in this mutant (Degenhardt *et al.*, 1998).

Arabidopsis has also been used as model system to test whether Al-induced genes can confer Al-resistance when overexpressed. Ezaki *et al.* (2000) overexpressed 9 Al-induced genes from *Arabidopsis*, tobacco, wheat and yeast and tested the resistance of transgenic plants to Al, oxidative stress and other metals such as Cd, Cu, Na, and Zn. Of the 9 genes tested, 4 were associated with increased resistance to Al in transgenic lines of *Arabidopsis*. These were *NtPox* (peroxidase), *NtPARB* (glutathione S-transferase), *NtGDI1* (GDP dissociation inhibitor) and *AtBCB* (blue copper binding protein), all of which have been implicated in response to oxidative stress. Overexpression of *NtPox*, *AtBCB* and *AtPox* also conferred resistance to diamide-induced oxidative stress, while overexpression of *NtPARB* conferred resistance to Cu and Na. A wheat cDNA encoding phosphatidylserine synthase, which conferred increased Al resistance when expressed in yeast, was also expressed in *Arabidopsis* (Delhaize *et al.*, 1999). Although expression of this enzyme is transcriptionally induced by Al in wheat root apices, the Al tolerance of transgenic *Arabidopsis* was not affected. These results suggest that there is not necessarily a link between Al-induction of genes and a role for their gene products in Al resistance, which may require appropriate regulation and localization of the Al-induced gene products or the presence of other enzymes.

Phenotype testing of yeast vacuolar ATPase mutants has shown that they are hypersensitive to Al and NaCl (Hamilton *et al.*, 2001a,b; Chapter Three; Chapter Four). This suggests that V-ATPase activity is required for Al and NaCl tolerance in yeast, as predicted by the energy balance hypothesis (Figure 3-1). To further test this hypothesis, I have examined the effect of altering V-ATPase expression in plants. Expression of a cDNA encoding the V-ATPase B subunit in the antisense orientation was used to downregulate V-ATPase expression in the model plant, *Arabidopsis thaliana*. This approach allowed me to directly test my hypothesis and further characterize the importance of the V-ATPase in mediating Al tolerance.

Although it would have been ideal to examine the effect of V-ATPase downregulation on Al tolerance in wheat, this was not practical due to the complexity of the wheat genome and the low efficiency of its *Agrobacterium*-mediated transformation (see for example Cheng *et al.*, 1997). *Arabidopsis* is commonly used as a model for plant biology because it has a comparatively small genome (125 Mb) which has been completely sequenced, it has been genetically well-characterized, a vast array of genetic resources are commercially available (http://www.Arabidopsis.org) and its small size allows simple manipulation of many plants on agar medium and in soil.

Due to the complex multisubunit structure of the V-ATPase (Stevens and Forgac, 1997; Chapter One), it was not feasible to construct transgenic plants overexpressing this enzyme. Therefore, the importance of V-ATPase activity in Al

tolerance was tested by expressing the cDNA encoding the B subunit in the antisense orientation. More specifically, I have used glucocorticoid-inducible expression of the V-ATPase B subunit cDNA in the antisense orientation to test the effect of downregulating V-ATPase activity on Al tolerance in *Arabidopsis*. If the energy balance hypothesis is correct, antisense expression should cause hypersensitivity to Al, as measured by inhibition of root elongation. If there is no discernable difference between transgenic and parent lines of *Arabidopsis*, then either the hypothesis can be rejected, which is unlikely based on our observations from wheat and yeast, or it must be modified to include other enzymes such as the vacuolar H^+-pyrophosphatase, which may be able to counteract decreased V-ATPase activity.

5.2 Materials and Methods

5.2.1 Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (all lines derived from ecotype C24) were surface sterilized in 20% (v/v) bleach, resuspended in 0.1% (w/v) agarose and plated on ½ MS medium (Murashige and Skoog basal medium, Sigma) containing 0.7% (w/v) phytagel, 2.5% (w/v) sucrose and 300 μg ml^{-1} timentin (SmithKline Beecham, Oakville ON) to limit bacterial growth. Following cold stratification at 4°C for 2 days, seeds were transferred to a controlled environment chamber at 20°C with 16 h light and 8 h dark. Alternatively, seeds were sown in soil (Redi-Earth, Grace Horticultural products, Ajax ON) and grown under the same conditions described

above. Soil-grown plants were fertilized weekly with 5 mM KNO_3, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.5 mM Fe·EDTA, 0.7 mM boric acid, 0.14 mM $MnCl_2$, 0.05 mM $CuSO_4$, 0.01 mM $ZnSO_4$, 0.007 mM $NaMoO_4$, 0.1 mM NaCl, 0.0001mM $CoCl_2$, 2.5 mM potassium phosphate buffer pH 5.5 (http://www.bch.msu.edu/pamgreen).

5.2.2 Transformation constructs

A cDNA encoding the V-ATPase B subunit from *Arabidopsis* (*At57*) was provided by Dr M. Manolson (Hospital for Sick Children, Toronto ON, Manolson *et al.*, 1988). It was excised from the pBluescript cloning vector and ligated into the glucocorticoid-inducible binary vector, pTA7002 (Aoyama and Chua, 1997), in the antisense orientation using unique *Xho*I and *Spe*I restriction enzyme sites. The presence of the desired insert in the resulting construct, pTA-At57a, was confirmed by sequencing. The pTA-At57a construct was transformed into *Agrobacterium tumefaciens* GV3101 by electroporation (T=2.5 kV, R=129 Ω, S=1.44 kV, BioRad Gene Pulser) and the presence of the construct confirmed by PCR. Primers used were C6 (5'-CACGCAACATCTTCCAGTCG-3'), at the 3' end of the transgene, and P8 (5'-ATTTGGAGAGGAACACGCTGA-3'), downstream of the *Spe*I cloning site.

5.2.3 Arabidopsis transformation

The floral dip method of *Arabidopsis* transformation (Clough and Bent, 1998) was used, with minor modifications. Seeds of *Arabidopsis thaliana* ecotype C24 were sown in soil in 15 cm diameter pots as described above. Following germination, plants were thinned to 10 per pot and grown until emergence of primary inflorescences. The primary inflorescences were removed and secondary

inflorescences allowed to reach approximately 5-15 cm tall with numerous flowers and buds. *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) carrying the binary vector of interest was grown overnight at 30°C in 500 ml YEP medium [10% (w/v) yeast extract, 10% (w/v) bacto-peptone, 5% (w/v) NaCl] containing 50 μg ml^{-1} kanamycin. The cell pellet was harvested by centrifugation and resuspended in 10% (w/v) sucrose, 0.05% (v/v) Silwet L-77 (Osi Specialties, Danbury CT) to a final OD_{600} of 0.8. The inflorescences were dipped into the *Agrobacterium* suspension for two 30 s intervals with gentle agitation. The floral dip was repeated one week later.

5.2.4 Selection and confirmation of transformants

T_1 seeds harvested from floral-dipped plants were sown on ½ MS medium containing 25 μg ml^{-1} hygromycin B (to select for transformants) and 300 μg ml^{-1} timentin as described above. Eight hygromycin-resistant seedlings (T_1) were transferred from tissue culture to soil after 4-6 weeks and matured in Arasystem 360 selfing tubes (BetaTech, Gent Belgium). T_2 seeds were harvested from individual T_1 plants to be used for experimental work. The presence of the transgene was tested by growth on medium containing hygromycin and by PCR, using the REDExtract-N-Amp plant PCR kit (Sigma) and primers specific to hygromycin phosphotransferase, P16 (5'-CATGTAGGAGGGCGTGGATA-3') and P17 (5'-CCTCCTCCATACAAGCCAAC-3'), and specific to the transgene, C6 (5'-CACGCAACATCTTCCAGTCG-3') and P8 (5'-ATTTGGAGAGGAACACGCTGA-3').

5.2.5 Transgene Copy Number Determination

For each of the 8 transgenic lines, 24 three-week old T_2 seedlings grown on ½ MS medium were transferred to ½ MS medium containing 50 μg mL^{-1} hygromycin B to determine the ratio of hygromycin-resistant to hygromycin-sensitive individuals. Plants were grown on hygromycin-containing medium for one week, when toxicity symptoms (chlorosis, death) were observed in control (C24) plants.

5.2.6 Root elongation measurements

Three-week old T_2 seedlings were transferred to ½ MS medium (1.4% phytagel) containing 0 or 30 μM dexamethasone (DEX) and either 100-500 μM $AlCl_3$, or 50-100 mM NaCl. The end of the longest root was marked on the plate with a marker and the plates were incubated vertically for 1 week (Ellis *et al.*, 1999). Root elongation from the starting mark was then measured. After the experiment, plants were tranferred to ½ MS medium containing 50 μg mL^{-1} hygromycin B to select for transgenic plants. Data points for nontransgenic plants were not included in the analysis. Plates containing 6 plants were used for each treatment and the experiment was performed three times.

5.3 Results

5.3.1 The Effect of Al on Root Elongation in Wild-type Arabidopsis

In order to quantify the effect of Al on wild-type *Arabidopsis*, the effect Al on root elongation was investigated. Three-week old seedlings of *Arabidopsis thaliana* ecotype C24 were transferred to ½ MS medium (pH 4.3) containing 0-1000 μM $AlCl_3$

and incubated vertically for one week. In the absence of Al, root length increased by 35.2 +/- 0.8 mm. Inhibition of root elongation by Al was observed at all concentrations tested, with virtually no elongation (0.8 +/- 0.1 mm) at 1000 μM $AlCl_3$ (Fig 5-1). In addition to inhibition of root elongation, concentrations of Al 500 μM or greater caused proliferation of lateral roots, which grew into the air. Based on these observations, Al concentrations of 100 μM and 500 μM were chosen for phenotype testing of transgenic plants. If plants expressing the B subunit of the V-ATPase in the antisense orientation are hypersensitive to Al, then the effect should be apparent at these concentrations.

5.3.2 The Effect of NaCl on Root Elongation in Wild-type Arabidopsis

The effect of NaCl on wild-type *Arabidopsis* was also investigated. Three-week old seedlings were transferred to ½ MS (pH 4.3) containing 0-300 mM NaCl and incubated vertically for one week (Fig 5-2). In the absence of NaCl, root length increased by 31.6 +/- 1.2 mm. Inhibition of root elongation was observed at all concentrations of NaCl tested, with complete inhibition at 200 and 300 mM (0 mm). Plants grown on ½ MS containing 200 mM NaCl showed minor chlorosis and club-shaped roots. At 300 mM NaCl, plants showed extensive chlorosis (data not shown). Since NaCl toxicity symptoms were severe at 200-300 mM NaCl, concentrations of 50 mM and 100 mM were chosen for phenotype testing of transgenic plants.

Fig 5-1. The effect of Al on root elongation in wild-type *Arabidopsis* (ecotype C24). Three-week old seedlings were grown vertically on ½ MS medium (pH 4.3) containing 0-1000 μM $AlCl_3$ for one week. Results shown are means +/- SE (n=6) and the results shown are representative of two independent experiments.

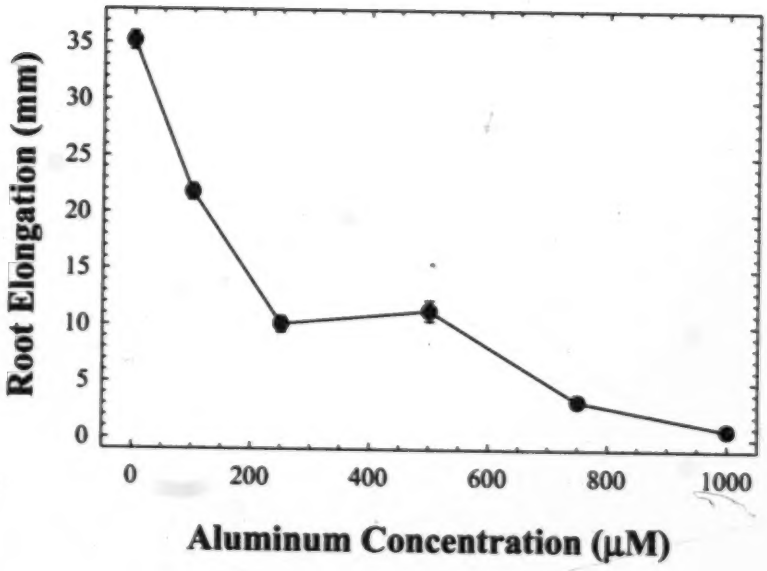
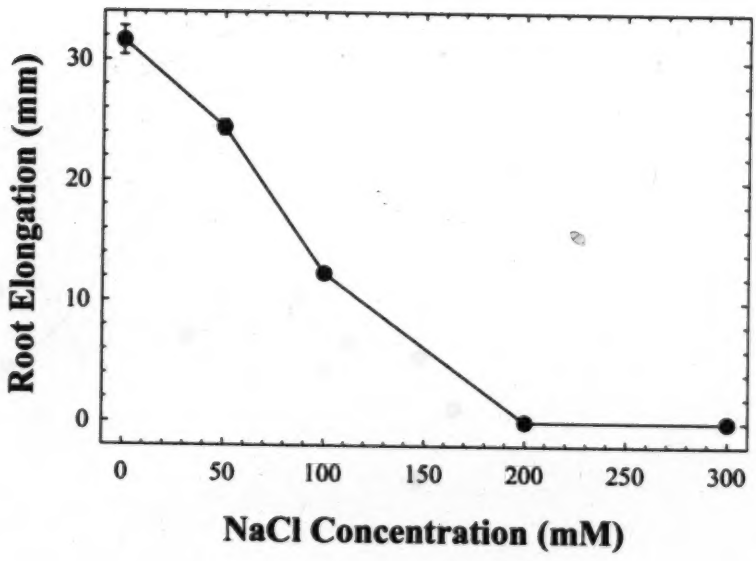

Root Elongation (mm)
35
30
25
20
15
10
5
0
0
200
400
600
800
1000
Aluminum Concentration (μM)

Fig 5-2. The effect of NaCl on root elongation in wild-type *Arabidopsis* (ecotype C24). Three-week old seedlings were grown vertically on ½ MS medium (pH 4.3) containing 0-300 mM NaCl for one week. Results shown are means +/- SE (n=6) and the results shown are representative of two independent experiments.


Root Elongation (mm)
30
20
10
0
0
50
100
150
200
250
300
NaCl Concentration (mM)

5.3.3 Production of Transgenic Arabidopsis Expressing the V-ATPase B Subunit in the Antisense Orientation

Since the V-ATPase is important in plant growth and development, a glucocorticoid-inducible promoter (Aoyama and Chua, 1997) was used to drive expression of the antisense RNA. The T-DNA of the binary vector pTA7002 (Fig 5-3) constitutively expresses a fusion protein (GVG) from the constitutive cauliflower mosaic virus 35S promoter. The fusion protein consists of the GAL4 DNA binding domain, the herpes viral protein VP16 transactivating domain and the receptor domain of the rat glucocorticoid receptor. The synthetic glucocorticoid, dexamethasone (DEX), binds to the GVG transcription factor and this complex activates transcription of the antisense RNA by binding to six copies of the GAL4 upstream activating sequence ($6xUAS_{GAL}$) located upstream of the transgene.

The antisense construct (Fig 5-3) was transformed into *Arabidopsis thaliana* ecotype C24 using the floral dip method (Clough and Bent, 1998). T_1 plants, selected by growth on medium containing hygromycin, were selfed and the resulting T_2 seeds were used for all experimental manipulations. To confirm that the T_2 plants were in fact transgenic, the presence of the hygromycin phosphotransferase gene and the transgene were determined by PCR. Genomic DNA was isolated from leaf discs of putative transgenic plants or parental plants for use in PCR reactions. The products of the PCR reactions were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (Fig 5-4). The 650 bp hygromycin phosphotransferase PCR product was present in all putative transgenic plants and

Fig 5-3. The T-DNA construct designed for glucocorticoid-inducible expression of the B subunit of the vacuolar ATPase (At57) in the antisense orientation (indicated by arrow). The NOS promoter controls expression of the selectable marker, hygromycin phosphotransferase (HPT). The constitutive cauliflower mosaic virus 35S promoter expresses a fusion protein (GVG) consisting of the GAL4 DNA binding domain, the herpes viral protein VP16 transactivating domain and the receptor domain of the rat glucocorticoid receptor, followed by the Rbc termination sequence. The synthetic glucocorticoid, dexamethasone (DEX), binds to the GVG transcription factor and this complex activates transcription of the antisense RNA by binding to six copies of the GAL4 upstream activating sequence ($6xUAS_{GAL}$) located upstream of the CMV 35S TATA box and the transgene. LB and RB denote the left and right borders of the T-DNA, respectively. The position of each component is given in bp. The diagram is not to scale.

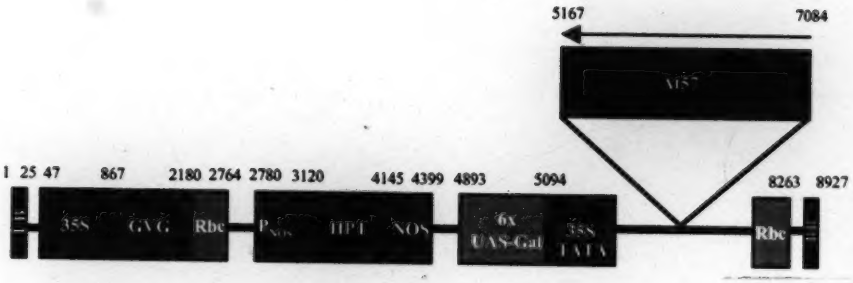
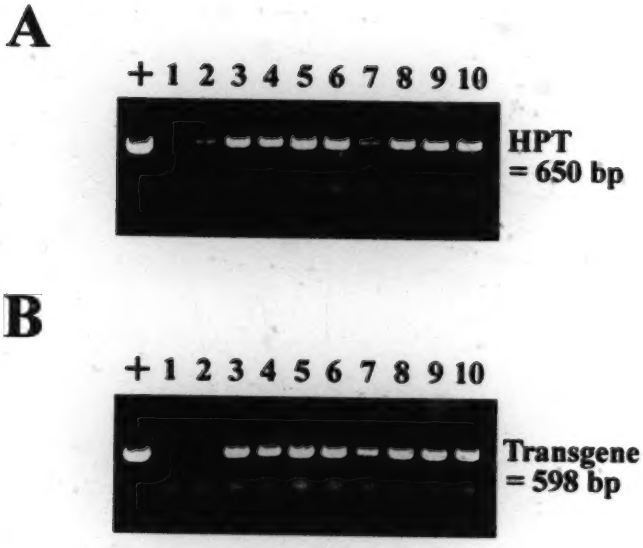

5167
7084
VP5
1 25 47
867
2180 2764 2780 3120
4145 4399 4893
5094
8263 8927
35S
GVG
Rbc
HPT
NOS
6x
UAS-Gal
35S
TATA
Rbc

Fig 5-4. Analysis of transgenic lines of *Arabidopsis* by PCR amplification of (A) a 650 bp fragment of hygromycin phosphotransferase and (B) a 598 bp fragment specific to the transgene. The positive control (+) was the plasmid used for transformation, pTA-At57a. Lane 1 is the parental line, C24, and lane 2 is the binary vector with no insert. Lanes 3-10 are independent transgenic lines 32, 33, 34, 36, 37, 38, 39 and 40, respectively. The results shown are representative of two independent experiments.


A
+ 1 2 3 4 5 6 7 8 9 10
HPT
= 650 bp
B
+ 1 2 3 4 5 6 7 8 9 10
Transgene
= 598 bp

absent in the parent, C24 (Fig 5-4A). The 598 bp transgene-specific PCR product was present in all eight antisense lines tested and absent in the parent and the vector controls (Fig 5-4B). These results confirmed that all of the antisense lines contained the construct in the correct orientation and that the transgene was absent in the parent and vector control lines.

The copy number of T-DNA inserts was determined by calculating the ratio of hygromycin-resistant to hygromycin-sensitive plants in the T_2 generation. Twenty-four T_2 seedlings from each independent transgenic line were transferred from ½ MS medium to ½ MS medium containing 50 μg mL^{-1} hygromycin B, which is toxic to parental plants. After 1 week, plants were scored as resistant or sensitive to hygromycin (Table 5-1). Hygromycin resistant plants were healthy and showed no apparent effects, while hygromycin sensitive plants were chlorotic and had ceased growth. The number of multiple T-DNA insertions in *Arabidopsis* was low. Of the eight lines tested here, four (33, 34, 38, 40) showed a 3:1 ratio, indicating that a single insertion event has occurred. Line 32 showed a 5:1 ratio, which also likely indicates that a single insertion event has occurred. Two lines (36 and 39) showed other ratios (11:1 and 24:0, respectively), indicating that multiple insertions have occurred. In line 37, which showed a ratio of approximately 1:1, homozygous transgenic individuals and nontransgenic individuals were likely killed by hygromycin. This may be the result of silencing of the hygromycin phosphotransferase gene. Transgenic lines 33, 34 and 40 were selected as the focus of phenotypic testing since they contained single copies of the T-DNA insert.

Table 5-1. Determination of copy number in transgenic *Arabidopsis thaliana* as measured by segregation of hygromycin resistance in T_2 progeny. Four week-old plants (24 from each line) were transferred to ½ MS medium containing 50 μg mL^{-1} hygromycin B for one week and scored as hygromycin resistant (no symptoms) or hygromycin sensitive (chlorotic, no growth).

Arabidopsis Line	Hygromycin-Resistant T_2 Progeny	Hygromycin-Sensitive T_2 Progeny	Ratio	Copy Number
C24 (parent)	0	24	0:24	0
32	20	4	5:1	1*
33	18	6	3:1	1
34	18	6	3:1	1
36	22	2	11:1	2
37	13	11	1:1	1 (silenced)
38	18	6	3:1	1
39	24	0	24:0	multiple
40	18	6	3:1	1

*Since a 5:1 ratio is nearer to 3:1 than 15:1 (expected for a double insertion event), this line is presumed to have a single insertion of the hygromycin phosphotransferase gene.

5.3.4 Phenotype Testing of Transgenic Arabidopsis

To examine the effect of downregulating V-ATPase expression on Al and NaCl tolerance, root elongation was measured. As demonstrated in Fig 5-1 and Fig 5-2, root elongation in three-week old *Arabidopsis* seedlings showed a dose-dependent response to both Al and NaCl. Aluminum concentrations of 100 μM and 500 μM and NaCl concentrations of 50 mM and 100 mM were chosen as exposure conditions based on these results. Dexamethasone at 30 μM was also included as a variable since it was required to induce transgene expression.

As shown in Fig 5-5, 5-7 and Fig 5-8, transgenic lines of *Arabidopsis* showed dexamethasone-dependent hypersensitivity to Al. The selected transgenic lines (33, 34 and 40) exhibited no dexamethasone toxicity, simplifying comparison between treatments. Some lines (32 and 37) showed significant inhibition of root elongation in the presence of dexamethasone (21% and 30%, respectively), so they were not included in the present discussion. At 100 μM Al, transgenic line 33 was inhibited a further 34% (from 20.8 mm to 13.8 mm) by the addition of dexamethasone, while lines 34 and 40 were inhibited a further 32% (from 21.3 mm to 14.5 mm) and 12% (from 14.5 mm to 12.8 mm), respectively. At 500 μM Al, addition of dexamethasone caused a further 30% decrease in root elongation in line 33 (from 13.8 mm to 9.7 mm) and further decreases of 51% (from 14.2 mm to 7.0 mm) and 34% (from 10.5 mm to 7.0 mm), respectively, in lines 34 and 40. In contrast, root elongation in C24 was inhibited by only 5% with the addition of dexamethasone (from 14.5 mm to 13.8 mm)

at 100 μM Al and not at all at 500 μM Al. Analysis of variance determined that the lines examined (C24 compared to transgenic), the presence of Al and dexamethasone caused significant effects ($P < 0.0001$). There were also significant interactions ($P < 0.01$) between line x aluminum and line x dexamethasone (Table 5-2A). In summary, the transgenic lines exhibited dexamethasone-dependent Al hypersensitivity.

Although the phenotypic data suggests that the transgene is being expressed, levels of the sense and antisense transcripts must be measured to confirm that the phenotype is caused by downregulation of V-ATPase expression. These experiments are in progress. Measurement of V-ATPase activity in dexamethasone-treated transgenic plants would provide additional evidence that the antisense strategy is effective.

Compared to the results observed for Al (Fig 5-5, Fig 5-7, Fig 5-8), the effect of downregulating V-ATPase expression on NaCl tolerance was variable (Fig 5-6, Fig 5-7, Fig 5-8). At 50 mM NaCl, addition of dexamethasone caused a 1% increase in root elongation (from 21.7 mm to 22.0 mm) in line 33, and decreases of 39% (from 23.8 mm to 14.5 mm) in line 34 and 3% (from 17.5 mm to 17.3 mm) in line 40. In C24, addition of dexamethasone at 50 mM NaCl caused root elongation to decrease by 26% (from 18.8 mm to 14.0 mm). At 100 mM NaCl, dexamethasone caused no additional decrease in root elongation in C24. In the transgenic lines, addition of dexamethasone caused decreases of 10% (from 17.2 mm to 15.5 mm) in line 33, 38% (from 15.7 mm to 9.8 mm) in line 34 and 2% (from 10.7 mm to 10.5 mm) in line 40.

Fig 5-5. The effect of transgene expression on Al resistance in *Arabidopsis*, measured by root elongation in the presence and absence of 30 μM dexamethasone (DEX). Three-week old T_2 seedlings were transferred to ½ MS medium (pH 4.3) containing 0 or 30 μM DEX and 0, 100 or 500 μM $AlCl_3$ and root elongation was measured after one week. Results shown are means +/- SE (n=6) and are representative of two independent experiments.

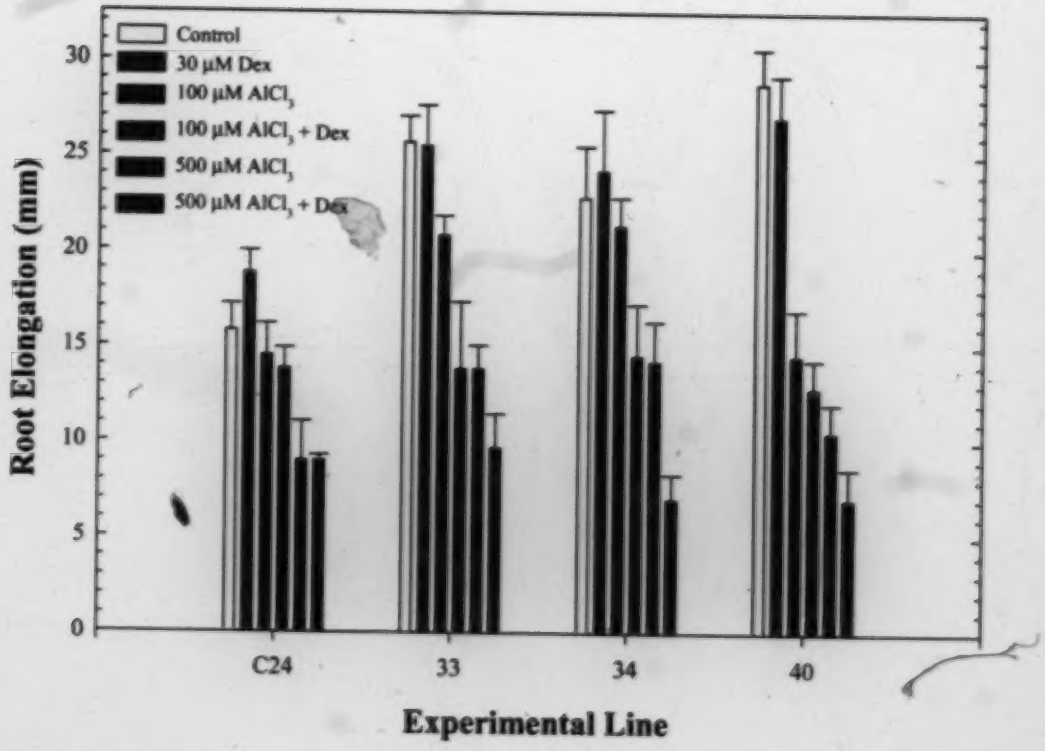

Control
30 µM Dex
100 µM AlCl3
100 µM AlCl3 + Dex
500 µM AlCl3
500 µM AlCl3 + Dex
Root Elongation (mm)
0
5
10
15
20
25
30
C24
33
34
40
Experimental Line

Fig 5-6. The effect of transgene expression on NaCl resistance in *Arabidopsis*, measured by root elongation in the presence and absence of 30 μM dexamethasone (DEX). Three-week old T_2 seedlings were transferred to ½ MS medium (pH 4.3) containing 0 or 30 μM DEX and 0, 50 or 100 mM NaCl and root elongation was measured after one week. Results shown are means +/- SE (n=6) and are representative of two independent experiments.

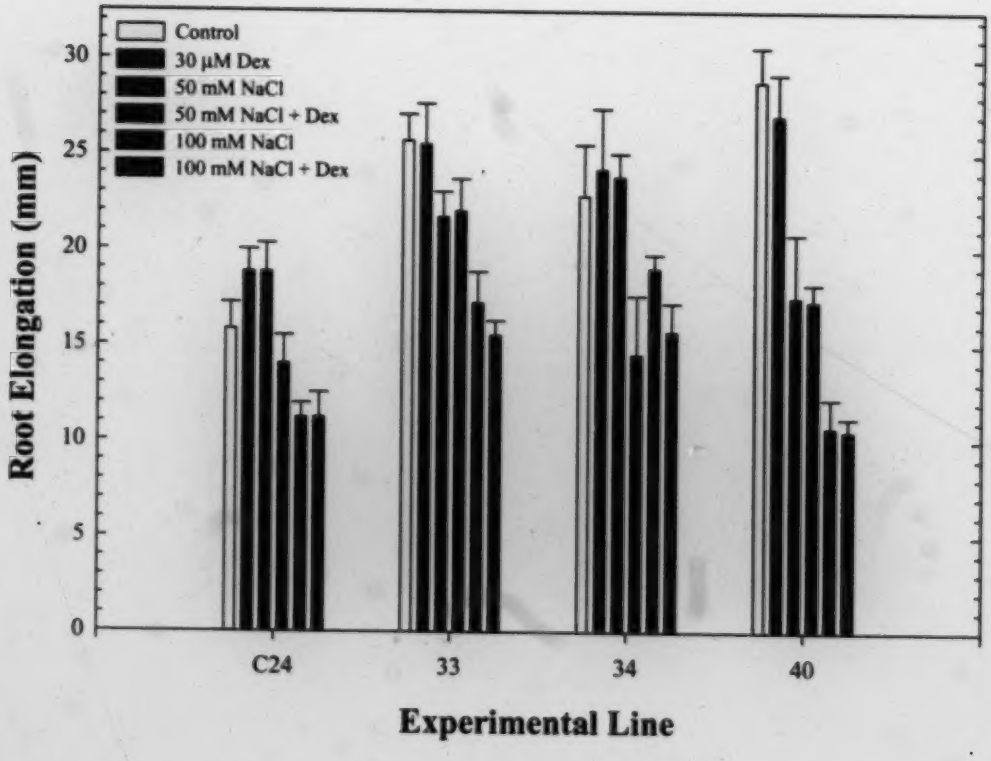

Control
30 µM Dex
50 mM NaCl
50 mM NaCl + Dex
100 mM NaCl
100 mM NaCl + Dex
Root Elongation (mm)
0
5
10
15
20
25
30
C24
33
34
40
Experimental Line

Fig 5-7. The effect of dexamethasone application on Al and NaCl resistance in wild-type *Arabidopsis thaliana* (ecotype C24). Three-week old T_2 seedlings were transferred to ½ MS medium (pH 4.3) containing 0 or 30 μM DEX and 0, 100 or 500 μM $AlCl_3$, and 50 or 100 mM NaCl for one week. Results shown are representative of two independent experiments.

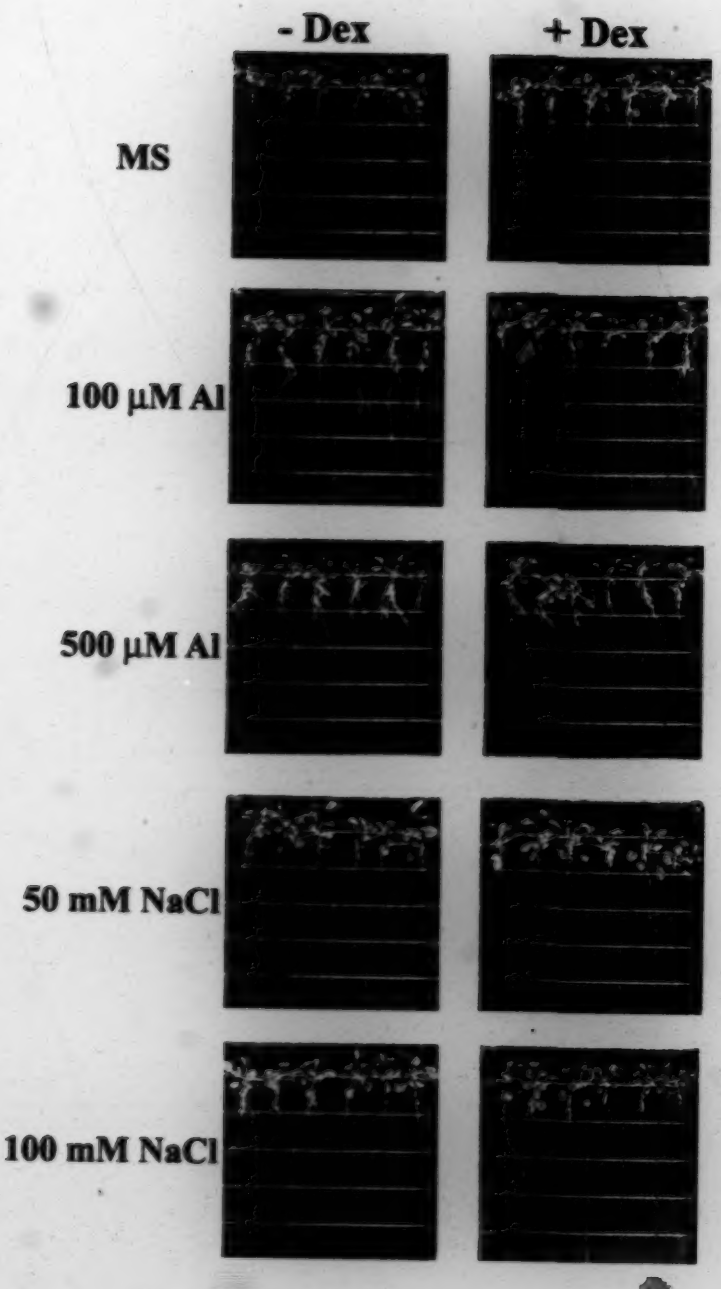

- Dex
+ Dex
MS
100 µM Al
500 µM Al
50 mM NaCl
100 mM NaCl

Fig 5-8. The effect of transgene expression on Al and NaCl resistance in transgenic *Arabidopsis thaliana* (line 34). Three-week old T_2 seedlings were transferred to ½ MS medium (pH 4.3) containing 0 or 30 μM DEX and 0, 100 or 500 μM $AlCl_3$, and 50 or 100 mM NaCl for one week. Results shown are representative of two independent experiments.

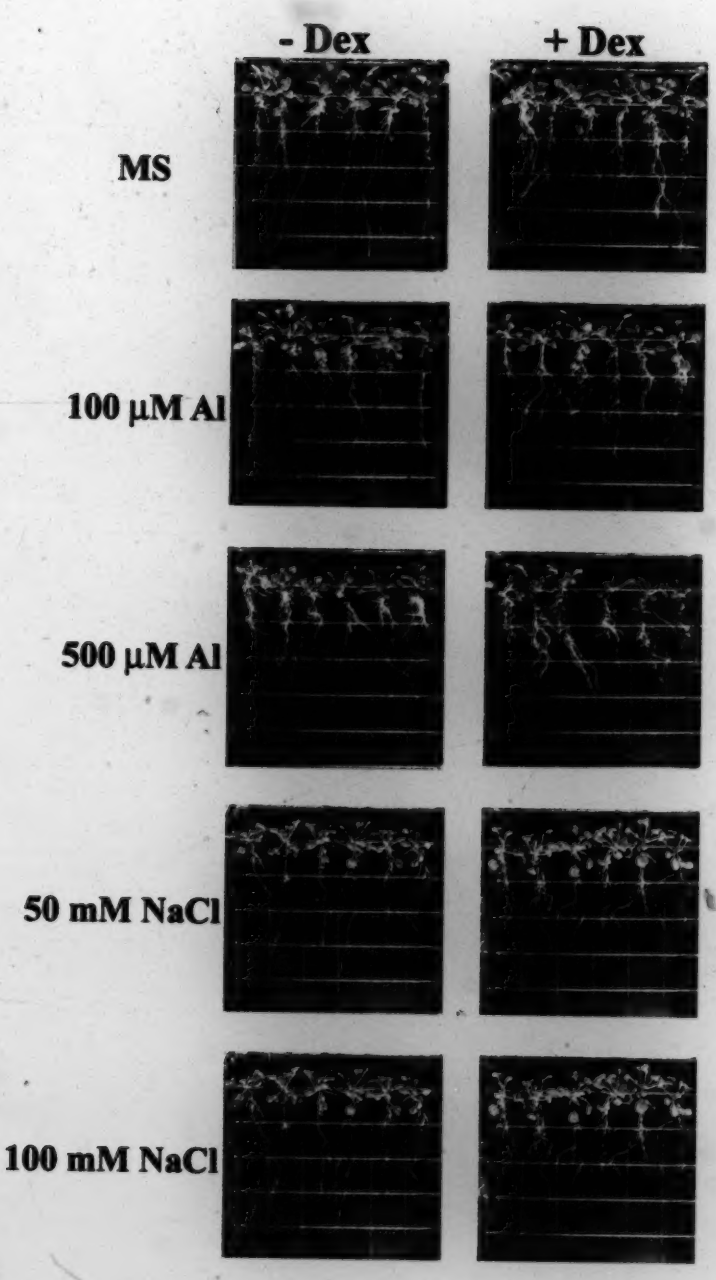

- Dex
+ Dex
MS
100 μM Al
500 μM Al
50 mM NaCl
100 mM NaCl

Table 5-2. Three factor analysis of variance of root elongation measurements of transgenic and parent (C24) lines in the presence of dexamethasone and/or (A) Al (B) NaCl. P values < 0.05 are considered significant, as indicated by asterisks (*).

	Variable	P Value
(A)	line	3.7×10^{-5}*
	aluminum	$< 1 \times 10^{-6}$*
	dexamethasone	1.7×10^{-5}*
	line x aluminum	1.4×10^{-3}*
	line x dexamethasone	7.0×10^{-3}*
	aluminum x dexamethasone	2.0×10^{-1}
	line x aluminum x dexamethasone	6.0×10^{-1}
(B)	line	$< 1 \times 10^{-6}$*
	salt	$< 1 \times 10^{-6}$*
	dexamethasone	1.7×10^{-2}*
	line x salt	1.4×10^{-3}*
	line x dexamethasone	3.2×10^{-1}
	salt x dexamethasone	9.5×10^{-1}
	line x salt x dexamethasone	1.0×10^{-1}

Analysis of variance suggests that the effect of transgene expression on NaCl tolerance is more variable than the effect on Al tolerance. The lines examined (C24 compared to transgenic) and the presence of NaCl had significant effects ($P < 1 \times 10^{-6}$) on root elongation. Dexamethasone had a weaker, but significant, effect ($P < 0.05$). There was a significant interaction ($P < 0.01$) between line x NaCl, but no significant interaction between line x dexamethasone (Table 5-2B). Although not as convincing as the results observed for Al, the results from line 34 indicate that downregulation of V-ATPase expression could potentially cause NaCl hypersensitivity.

5.4 Discussion

Previous investigations of Al tolerance using transgenic technology in *Arabidopsis* have overexpressed many Al-induced genes in an effort to cause a phenotypic change (Ezaki *et al.*, 2000; Richards *et al.*, 1998). The moderate success of this approach indicates that the complex interactions involved in Al tolerance must be taken into consideration when designing transgenic experiments. The approach described here is hypothesis-driven, based on previous work in wheat (Hamilton *et al.*, 2001c; Chapter Two) and yeast (Hamilton *et al.*, 2001a, 2001b; Chapter Three, Chapter Four). Results from my previous work indicated that V-ATPase expression is induced by Al stress in an Al-resistant cultivar of wheat (Hamilton *et al.*, 2001c; Chapter Two). The V-ATPase is also required for Al and NaCl resistance in yeast (Hamilton *et al.*, 2001a, 2001b; Chapter Three, Chapter Four). Based on these

results, I predicted that downregulation of V-ATPase activity would cause Al and NaCl hypersensitivity in transgenic *Arabidopsis*.

According to the energy balance hypothesis (Fig 3-1), downregulating V-ATPase activity should cause hypersensitivity to Al. The results presented here are consistent with this hypothesis, since transgenic plants show dexamethasone-dependent Al hypersensitivity. It is unknown whether this hypersensitivity is caused by impairment of a pH homeostatic mechanism or by impaired Al compartmentation in the vacuole. Although it is tempting to speculate about Al sequestration, no conclusions can be drawn in the absence of data on Al localization. The V-ATPase may have a role in pH homeostasis during Al stress, similar to the *alr104* mutant characterized by Degenhardt *et al.* (1998). This mutant exhibits enhanced H^+ influx across the plasma membrane in response to Al stress, resulting in alkalinization of the rhizosphere. Increased activity of the V-ATPase could result in alkalinization of the cytoplasm, which would reduce the solubility and/or toxicity of intracellular Al, and would also prevent acidosis caused by impairment of the plasma membrane H^+-ATPase by Al (Matsumoto, 1988; Sasaki *et al.*, 1995; Widell *et al.*, 1994; Hamilton *et al.*, 2001a).

Unlike their response to Al, the response of the transgenic plants to NaCl was variable. Overexpression of the Na^+/H^+ antiporter (Apse *et al.*, 1999) or the vacuolar H^+-pyrophosphatase (Gaxiola *et al.*, 2001) conferred increased NaCl tolerance in transgenic *Arabidopsis*. This suggests that maintenance of the tonoplast membrane potential contributes to NaCl tolerance by providing the necessary proton motive

force for sequestration of Na^+ in the vacuole. It is possible that the conditions used here were not optimal for observing a phenotypic difference, since Gaxiola *et al.* (2001) used a soil system and watered their transgenic plants with saline solutions. These conditions are different from the tissue culture conditions used here, so it would be interesting to test our transgenic plants in soil using their regimen. It would also be a powerful test of the energy balance hypothesis to use plants overexpressing the vacuolar H^+-pyrophosphatase (Gaxiola *et al.*, 2001) in the assays described here, since one would predict that they would be tolerant to Al as well as to NaCl.

Since the vacuolar H^+-ATPase is required for growth and development, the glucocorticoid-responsive transcriptional induction system was used to control antisense expression. This system has the advantages of localized, inducible expression of transgenes at variable levels. It has been used successfully to control expression of the luciferase gene (Aoyama and Chua, 1997) as well as many other genes (for example McNellis *et al.*, 1998; Lally *et al.*, 2001; Okamoto *et al.*, 2001; Pautot *et al.*, 2001) in transgenic *Arabidopsis*. However, this system has limitations. In certain transgenic lines, the glucocorticoid-inducible system can cause severe growth defects and induce defense-related genes in transgenic *Arabidopsis* (Kang *et al.*, 1999). This was not observed with the transgenic lines used here. This suggests that the transgenic lines used, as well as concentration and means of application (liquid medium, agar or spraying) of dexamethasone may be important variables to be considered when analyzing data from this system. It is also essential to include

dexamethasone treatment as an experimental control to rule out nonspecific dexamethasone-induced effects.

In summary, this chapter describes the design, construction and phenotypic testing of transgenic Arabidopsis expressing a cDNA encoding the B subunit of the V-ATPase in the antisense orientation. Induction of transgene expression by dexamethasone causes hypersensitivity to Al, as predicted by the energy balance hypothesis. Interestingly, transgenic plants showed a variable phenotype with respect to NaCl tolerance. Further investigation will need to determine why certain lines exhibit NaCl hypersensitivity while others do not so that the role of the V-ATPase in NaCl tolerance can be determined.

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6. Concluding Discussion

In this thesis, the role of the vacuolar ATPase in aluminum resistance has been examined in wheat (*Triticum aestivum* L.), yeast (*Saccharomyces cerevisiae*) and thale cress (*Arabidopsis thaliana*). Earlier research from our laboratory identified two 51 kD, Al-induced proteins from an Al-resistant cultivar of wheat (PT741), which were newly synthesized upon administration of Al stress (Basu *et al.*, 1994). Induction of these proteins segregated with Al-resistance in a segregating population derived from a cross between Al-resistant PT741 and Al-sensitive Katepwa (Taylor *et al.*, 1997). These observations indicated that Al-induction of the 51 kD proteins may be an adaptive response involved in mediating Al resistance.

The 51 kD, Al-induced proteins were purified, sequenced and found to be subunits of the vacuolar H^+-ATPase (V-ATPase) and the mitochondrial F_1F_0-ATPase. Their identities were further confirmed by measuring ATPase activity in Al treated roots of PT741, where Al induced both V-ATPase and F_1F_0-ATPase activities in a dose-dependent manner. Levels of the transcript encoding the B subunit of the V-ATPase also increased with Al treatment, although levels of the transcript encoding the α subunit of the F_1F_0-ATPase were unaffected by Al at the levels tested. Interestingly, increased V-ATPase and F_1F_0-ATPase activities were observed only in PT741 and not in the other Al-resistant and Al-sensitive cultivars of wheat tested. In contrast, inhibition of the plasma membrane H^+-ATPase was observed in all cultivars. This suggests that induction of the V-ATPase and F_1F_0-ATPase, thought to be an

adaptive response to Al stress, is specific to this cultivar. This may prove useful in future breeding programs for Al resistance.

Based on the observation that the V-ATPase and the F_1F_0-ATPase are induced by Al, a testable hypothesis describing a possible role for these enzymes in mediating Al resistance was developed (Fig 3-1). Briefly, this hypothesis predicts that the V-ATPase, using ATP generated by the F_1F_0-ATPase, is responsible for preventing cytoplasmic acidosis caused by Al-inhibition of the plasma membrane H^+-ATPase. In addition to protecting sensitive cytoplasmic proteins from pH changes, prevention of cytoplasmic acidosis is also required to allow continued tonoplast transmembrane transport. Whether this transport involves sequestration of an unknown Al species remains to be determined. Considering recent advances in analysis of Al uptake in single cells, it may be possible to address this question directly in the near future.

It is not presently possible to manipulate gene expression in monocots using transgenic technology without highly labour-intensive techniques. Yeast, however, is amenable to relatively simple genetic manipulation, so it was used as a model system to test my hypothesis. Yeast strains with knockout mutations in the genes encoding the A and B subunits of the V-ATPase, but not those with mutations in the genes encoding the α and β subunits of the F_1F_0-ATPase, exhibited dramatic hypersensitivity to Al in comparison to the isogenic wild-type parent and complemented strains. This provided support for the hypothesis that V-ATPase activity is involved in mediating Al resistance, even in a different species. This hypothesis could also be tested using yeast strains overexpressing the vacuolar H^+-

pyrophosphatase (Gaxiola *et al.*, 1999). Overexpession of this single protein is analagous to overexpression of the multisubunit V-ATPase. According to my hypothesis, yeast strains overexpressing this enzyme should exhibit enhanced Al resistance. Phenotype testing of this strain is in progress.

Characterization of wild-type yeast revealed that V-ATPase activity, transcript and protein levels were unchanged by the addition of Al, in contrast to the Al-induction of V-ATPase activity and transcript levels observed in wheat. Mitochondrial F_1F_0-ATPase activity was induced by Al, consistent with observations from wheat. The differences in V-ATPase expression in wheat and yeast are reminiscent of the V-ATPase activity differences between Al-resistant PT741 and Al-sensitive cultivars of wheat. This may lend further support to the hypothesis that V-ATPase activity is involved in Al resistance.

Genetic studies have shown that the yeast V-ATPase contributes to NaCl tolerance, presumably by establishing the tonoplast transmembrane potential required for sequestration of Na^+ in the vacuole. This has been convincingly demonstrated by overexpressing the *Arabidopsis* vacuolar H^+-pyrophosphatase in yeast (Gaxiola *et al.*, 1999) and *Arabidopsis* (Gaxiola *et al.*, 2001), where it confers increased NaCl tolerance. My observation that yeast strains with knockout mutations in the genes encoding the A and B subunits of the V-ATPase are hypersensitive to NaCl lends further support to the argument that vacuole function is required for NaCl tolerance.

Although NaCl tolerance in yeast has been intensively investigated, the response of ATPases to NaCl in wild-type yeast had not been previously

characterized. Therefore, the scope of my research was expanded to include analysis of ATPase expression in response to NaCl stress with the hope that parallels could be drawn with Al stress. In contrast to the constant levels observed with increasing Al concentrations, V-ATPase activity, protein and transcript levels increased with the addition of NaCl. In another interesting contrast, induction of the F_1F_0-ATPase was also observed, but to a lesser extent. The reason that F_1F_0-ATPase expression is more highly induced by Al in yeast is unknown, but it is possible that additional energy is required to combat Al stress. In the case of NaCl, energy is required because to sequester Na^+ in the vacuole and to export Na^+ from the cell by active transport across the plasma membrane (Marquez and Serrano, 1996). Although NaCl induces F_1F_0-ATPase expression, yeast strains with knockout mutations in the genes encoding the α and β subunits of the F_1F_0-ATPase were not hypersensitive to NaCl. This suggests that for NaCl, like Al, the V-ATPase is more critical in stress resistance. The genetic approach used here may point towards a new direction in Al stress research. A program of systematically studying components thought to be involved in Al resistance using yeast knockout mutants has been proposed as a means of dissecting this complex stress response. This approach will allow us to investigate the involvement of individual components or pathways in Al resistance in a simple model system.

Since experiments performed with yeast supported my hypothesis that the V-ATPase is required for Al and NaCl tolerance, the next step was to test whether or not altering the expression of the V-ATPase could affect Al and NaCl tolerance in plants.

Arabidopsis thaliana was selected as a model plant for these experiments because it can be easily transformed, unlike wheat (Cheng *et al.*, 1997). Inhibition of V-ATPase expression was achieved using a glucocorticoid-inducible antisense approach, which has the advantage of temporal and spatial control of antisense RNA expression (Aoyama and Chua, 1997) and because phenotypic screening is relatively simple. Although antisense inhibition of V-ATPase expression was not measured directly, T_2 transgenic lines exhibited glucocorticoid-dependent Al hypersensitivity. Ongoing experiments are investigating V-ATPase expression by measuring enzyme activity, antisense and sense RNA levles, in dexamethasone-treated roots. Some lines were also hypersensitive to NaCl, although this aspect of their phenotype was not as clear. These results could be supplemented by testing the phenotype of transgenic *Arabidopsis* expressing the vacuolar H^+-pyrophosphatase. Overexpression of this enzyme in *Arabidopsis*, which is analogous to overexpression of the V-ATPase, causes enhanced NaCl tolerance in transgenic lines (Gaxiola *et al.*, 2001). It would be informative to obtain these lines and test their Al and NaCl tolerance in the root elongation system.

In future, it would be interesting to determine the precise mechanism by which increased V-ATPase activity mediates Al resistance. Is V-ATPase activity required to allow transmembrane transport of an Al species, analagous to how it is involved in NaCl tolerance? It is also possible that the tonoplast transmembrane potential is required to allow transport of signaling molecules, such as calcium, which then trigger other downstream events. Finally, it is possible that increased V-ATPase

activity is simply a mechanism of pH control. Comparing vacuolar accumulation of Al in Al-resistant PT741 and Al-sensitive wheat cultivars could test the first of these possibilities. However, the rare ^{26}Al isotope and specialized microextraction techniques would need to be used to measure low levels of Al within root tip cells (Taylor *et al.*, 2000). Even with these techniques, the required sensitivity may not be available. Examining Ca^{2+} fluxes during Al stress could determine whether or not Ca^{2+} release from vacuolar stores exhibits a different pattern in PT741 than in other wheat cultivars. The simplest of these possibilities, that the V-ATPase acts as an intracellular "pH-stat", could be tested using microelectrodes or indicator dyes to measure the cytoplasmic pH of individual cells of PT741 and other wheat cultivars during Al stress. The above list is not exhaustive, but it illustrates the additional layers of complexity which remain to be unraveled by future research. It is possible that one or more of these mechanisms, or a different mechanism not discussed here, is responsible for V-ATPase mediated Al-resistance.

In conclusion, in this thesis I have: 1) identified three Al-induced proteins as the B subunit of the vacuolar H^+-ATPase and the α and β subunits of the mitochondrial F_1F_0-ATPase, 2) demonstrated that V-ATPase and F_1F_0-ATPase activities are induced by Al in Al-resistant PT741 and not in other Al-resistant or Al-sensitive wheat cultivars tested, 3) developed a testable hypothesis describing a possible mechanism by which increased V-ATPase and F_1F_0-ATPase activities could mediate Al resistance, 4) verified this hypothesis using knockout mutants of yeast and 5) transgenic Arabidopsis and 6) expanded this hypothesis by analogy to NaCl

tolerance. Further support for my hypothesis awaits future phenotypic testing of Al resistance in yeast and transgenic plants overexpressing the vacuolar H^+-pyrophosphatase, and determination of the mechanism by which increased V-ATPase activity mediates Al resistance.

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